Catabolism of biomass-derived sugars in fungi and metabolic engineering as a tool for organic acid production

Outi Koivistoinen
Catabolism of biomass-derived sugars in fungi and metabolic engineering as a tool for organic acid production

Outi Koivistoinen

Department of Food and Environmental Sciences
Faculty of Agriculture and Forestry,
University of Helsinki, Finland

Thesis for the degree of Doctor of Science (Agriculture and Forestry) to be presented, with due permission for public examination and criticism in Auditorium 1041 of the Viikki Biocenter (Viikinkaari 5), at the University of Helsinki, on the 8th of November at 12 noon.
Catabolism of biomass-derived sugars in fungi and metabolic engineering as a tool for organic acid production

Biomassaperäisten sokerien katabolia eukaryoottimikrobeissa ja metaboliamuokkauksen hyödyntäminen organisten happojen tuotossa. Outi Koivistoinen.
Espoo 2013. VTT Science 43. 86 p. + app. 56 p.

Abstract

The use of metabolic engineering as a tool for production of biochemicals and biofuels requires profound understanding of cell metabolism. The pathways for the most abundant and most important hexoses have already been studied quite extensively but it is also important to get a more complete picture of sugar catabolism. In this thesis, catabolic pathways of L-rhamnose and D-galactose were studied in fungi. Both of these hexoses are present in plant biomass, such as in hemicellulose and pectin. Galactoglucomannan, a type of hemicellulose that is especially rich in softwood, is an abundant source of D-galactose. As biotechnology is moving from the usage of edible and easily metabolisable carbon sources towards the increased use of lignocellulosic biomass, it is important to understand how the different sugars can be efficiently turned into valuable biobased products.

Identification of the first fungal L-rhamnose 1-dehydrogenase gene, which codes for the first enzyme of the fungal catabolic L-rhamnose pathway, showed that the protein belongs to a protein family of short-chain alcohol dehydrogenases. Sugar dehydrogenases oxidising a sugar to a sugar acid are not very common in fungi and thus the identification of the L-rhamnose dehydrogenase gene provides more understanding of oxidative sugar catabolism in eukaryotic microbes. Further studies characterising the L-rhamnose cluster in the yeast Scheffersomyces stipitis including the expression of the L-rhamnionate dehydratase in Saccharomyces cerevisiae finalised the biochemical characterisation of the enzymes acting on the pathway. In addition, more understanding of the regulation and evolution of the pathway was gained.

D-Galactose catabolism was studied in the filamentous fungus Aspergillus niger. Two genes coding for the enzymes of the oxido-reductive pathway were identified. Galactitol dehydrogenase is the second enzyme of the pathway converting galactitol to L-xyl-o-3-hexulose. The galactitol dehydrogenase encoding gene ladB was identified and the deletion of the gene resulted in growth arrest on galactitol indicating that the enzyme is an essential part of the oxido-reductive galactose pathway in fungi. The last step of this pathway converts D-sorbitol to D-fructose by sorbitol dehydrogenase encoded by sdhA gene. Sorbitol dehydrogenase was found to be a medium chain dehydrogenase and transcription analysis suggested that the enzyme is involved in D-galactose and D-sorbitol catabolism.
The thesis also demonstrates how the understanding of cell metabolism can be used to engineer yeast to produce glycolic acid. Glycolic acid is a chemical, which can be used for example in the cosmetic industry and as a precursor for biopolymers. Currently, glycolic acid is produced by chemical synthesis in a process requiring toxic formaldehyde and fossil fuels. Thus, a biochemical production route would be preferable from a sustainability point of view. Yeasts do not produce glycolic acid under normal conditions but it is a desired production host for acid production because of its natural tolerance to low pH conditions. As a proof of concept, pure model substrates, e.g. D-xylose and ethanol, were used as starting materials for glycolic acid production but the knowledge can be further applied to an expanded substrate range such as biomass derived sugars. Already the introduction of a heterologous glyoxylate reductase gene resulted in glycolic acid production in the yeasts S. cerevisiae and Kluyveromyces lactis. Further modifications of the glyoxylate cycle increased the production of glycolic acid and it was successfully produced in bioreactor cultivation.

The challenge of biotechnology is to produce high value products from cheap raw materials in an economically feasible way. This thesis gives more basic understanding to the topic in the form of new information regarding L-rhamnose and D-galactose metabolism in eukaryotic microbes as well as provides an example on how cell metabolism can be engineered in order to turn the cell into a cell factory that is able to produce a useful chemical.

Keywords: rhamnose, galactose, Scheffersomyces stipitis, Aspergillus niger, Saccharomyces cerevisiae, metabolic engineering, glyoxylate cycle, glycolic acid
Biomassaperäisten sokerien katabolia eukaryoottimikrobeissa ja
metaboliamuokkauksen hyödyntäminen orgaanisten happojen tuotossa

Catabolism of biomass-derived sugars in fungi and metabolic engineering as a tool for
organic acid production. Outi Koivistoinen. Espoo 2013. VTT Science 43. 86 s. + liit. 56 s.

Tiivistelmä

Metaboliamuokkauksen käyttäminen biokemikaalien ja biopolttoaineiden tuoton
 työkaluna vaatii syvällistä solun metabolian ymmärrystä. Yleisimpien ja tärkeimpien
 heksosien, kuten D-glukoosin ja D-fruktoosin, metabolia on jo melko hyvin tutkittu
 alue, mutta kokonaiskuvan hahmottamiseksi laajempi sokerimetabolian ymmärta-
 minen on tärkeää. Tässä väitöskirjassa on tutkittu kahden sokerin,
L-ramnoosin ja D-galaktoosin, kataboliareittejä hiivossa ja filamenttihomeissa. Näitä molempia
heksosisokereita esiintyy kasvibiomassassa, kuten hemiselluloosassa ja pek-
tiinisissä. Galaktoglukomannaani on hemiselluloosatyypin, jossa on paljon
 D-galaktoosia ja jota on erityisesti havupuissa. Bioteknologian sovelluksissa yrte-
tään parhaillaan siirtyä ruoaksi kelpaavien helposti hyödynnettävien raaka-
aineiden käytöstä kohti lignoselluloosaperäisten biomassojen hyödyntämistä, joten
on hyvin tärkeää ymmärtää, miten erilaiset sokerit pystytään muuntamaan hyödyllisiiksi
biopohjaisiksi tuotteiksi.

Ensimmäisen eukaryoottimikrobiperäisen L-ramnoosi 1-dehydrogenaasi-entsyymiä
koodaavan geenin identifiointi osoitti, että L-ramnoosi 1-dehydrogenaasi-entsyymi
kuuluu lyhyketjuisten alkoholidehydrogenaasien proteiiniperheeseen. Sokeride-
hydrogenaasit, jotka hapettavat sokereita sokerihapoksi, eivät ole yleisiä euka-
ryoottimikrobeissa, joten L-ramnoosi 1-dehydrogenaasigenen identifiointi tuo lisää
ymmärrystä eukaryoottimikrobioiden oksidatiivisesta sokerikataboliasta. Työitä jatkettiin
karakterisoimalla Scheffersomyces stipitis -hiivan L-ramnoosiklusteri. Työssä eks-
pressoi L-ramnooattidehydrogenaasigenen Saccharomyces cerevisiae -hiivassa ja
täten viimeisteltiin L-ramnoosireitin entsymien biokemiallinen karakterisointi. Li-
sääsi työ lisäsi ymmärrystä reitin säätelystä ja evoluutiosta.

D-Galaktoosityö tehtiin Aspergillus niger -homeessa ja siinä identifiointiin kaksi
oksido-reduktiivisen galaktosimetaboliareitin entsyymiä koodaavaa geeniiä. Ga-
laktitidehydrogenaasi, joka muuntaa galaktitoliin L-ksylo-3-heksuloosiksi, on reitin
toen entsyymi. Galaktitidehydrogenaasia koodava geeni  ladhB identifiointiin ja
genin deleetion huomattiin aiheuttavan fenotyypin, joka ei kasva galaktitoliilla. Tämä
osoittaa, että kyseen entsyymi on olennainen osa kyseistä metaboliareittia
eukaryoottimikrobeissa. Reitin viimeinen entsyymi, sdtA-geenin koodaama sorbi-
tolidehydrogenaasi, muuntaa D-sorbitolia D-fruktoosiksi. Sorbitolidehydrogenaasi
identifiointiin keskitetujukseksi dehydrogenaasiksi ja transkriptioanalyysi osoitti,
ett entsyymi osallistuu sekä D-galaktoosin että D-sorbitolin kataboliaan.

Väitöskirja myös demonstroi, kuinka solun metabolian ymmärrystä voidaan käyttää
hyödyksi metabolismuokkauksessa ja näin saada hiiva tuottamaan glykolihappoa.

Biotekniikan haaste on tuottaa halvoista raaka-aineista hyödyllisiä biopohjaisia tuotteita taloudellisesti kannattavalla tavalla. Tämä välttäisi kriitikko tuo lisää ymmärrystä aiheeseen tuomalla uutta tietoa L-rhamnoosin ja D-galaktoosin metabolisista eukaryootimikrobeissa ja yhtä lailla antaa esimerkin, kuinka metabolista ymmärtämystä voidaan käyttää solun muokkauksessa hyödyllistä kemikaalia valmistavaksi solutehtäksi.

Avainsanat: rhamnose, galactose, *Scheffersomyces stipitis*, *Aspergillus niger*, *Saccharomyces cerevisiae*, metabolic engineering, glyoxylate cycle, glycolic acid
Preface

This study was carried out at the VTT Technical Research Center of Finland in the Metabolic Engineering team. Financial support was provided by the Academy of Finland (Center of Excellence, White Biotechnology – Green Chemistry 2008 – 2013; project number 118573 and SA Tools 2; project number 126506) and by the Forestcluster Ltd. Future Biorefinery programme, and travel funding by the BIOREGS Graduate School for Biomass Refining, and were greatly appreciated.

I sincerely thank Vice President, Professor Anu Kaukovirta-Norja, former Technology Manager Dr. Tiina Nakari-Setälä and Technology Manager Docent Kirsi-Maaria Oksman-Caldentey for providing the opportunity to prepare this thesis and for providing excellent working facilities. Team Leader Dr. Laura Ruohonen is delightfully thanked for her supportive attitude towards the work.

I wish to express my deepest gratitude to my supervisor Docent Peter Richard for guidance and encouragement in all situations. Peter’s devotion to science is admirable and has offered an invaluable source of new ideas during the years spent working on this thesis. Dr. Dominik Mojzita is warmly thanked for supervising the *Aspergillus* work and suggesting that it be included in this thesis. Dominik’s enthusiasm for *Aspergillus* research motivated me to learn a lot about filamentous fungi and how to work with them. Research Professor Merja Penttilä is thanked for scientific guidance and her encouraging attitude towards the work presented in this thesis.

I thank Professor Helena Nevalainen and Associate Professor Alexander Frey for careful pre-examination of the thesis and for their useful comments about how to improve it. I wish to thank Professor Annele Hatakka for being responsive to my sometimes quite urgent schedules concerning the thesis and PhD studies during the past spring. I am grateful to Dr. Andrew Conley for revising the language of the thesis, and for his encouraging comments on how to improve it. He and Dr. Marilyn Wiebe are also thanked for providing linguistic advice for some of my manuscripts.

My warmest thanks to my co-authors, Professor Merja Penttilä, Professor Thomas W. Jeffries, Docent Peter Richard, Docent Hannu Maahemo, Dr. Martina Andberg, Dr. Mikko Arvas, Dr. Dorothee Barth, Dr. Harry Boer, Dr. Jennifer R. Headman, Dr. Satu Hilditch, Dr. Dominik Mojzita, Dr. Juha-Pekka Pitkänen, Dr. Laura Ruohonen, Dr. Sanni Voutilainen, Joosu Kuivanen and Heidi Turkia, for their contributions to the research and writing of the manuscripts. Without their input this work would not have been possible.
My sincere thanks to everyone working in VTT’s yeast-mould lab for the support and help I have received. It has been a joy to work in the enthusiastic and friendly atmosphere of the lab! In addition I wish to thank people in the other labs of VTT’s Tietotie 2 building in which I carried out occasional experiments. The help provided and advice given was essential for the bioreactor cultivations and protein purification work. Especially I wish to thank Ali Grundström, Toni Paasikallio and Marjo Öster for skilful technical assistance.

I am grateful to Mervi for being an excellent officemate through all the years spent working on this thesis. Mervi’s encouraging and helpful attitude has helped to prevent and solve many problems. I warmly thank my colleagues and friends Yvonne, Mari, Joosu, Kiira, Eero, Pekka and Mira. Lunches and coffee breaks with you have been important breaks for me. Yvonne, Mari and Joosu, thank you for all the good discussions we have had about PhD studies and work related subjects. I greatly value you for always being willing to help and for sharing not only your work and study related matters, but also for being my friends outside work hours and lab facilities. Kiira and Stefan, it has been great that we have been able to keep in touch and meet every now and then, even though the distance between us has increased since the time when we were all working at VTT.

Finally I would like to thank my friends and family for support. Especially I am indebted to my husband Ossi who has never complained about my working hours, but instead has always been willing to help with my computer and figure editing problems – with this thesis also. Thank you for love and encouragement through the years.

Espoo, October 2013

Outi
Academic dissertation

Supervisors
Docent Peter Richard
VTT Technical Research Centre of Finland
Espoo, Finland

Dr. Dominik Mojzita
VTT Technical Research Centre of Finland
Espoo, Finland

Professor Merja Penttilä
VTT Technical Research Centre of Finland
Espoo, Finland

Reviewers
Associate Professor Alexander Frey
Department of Biotechnology and Chemical Technology
Aalto University, Finland

Professor Helena Nevalainen
Department of Chemistry and Biomolecular Sciences
Macquarie University, Australia

Opponent
Professor Marie-Francoise Gorwa-Grauslund
Center for Chemistry and Chemical Engineering
Lund University, Sweden

Custos
Professor Annele Hatakka
Department of Food and Environmental Sciences
University of Helsinki, Finland
List of publications

This thesis is based on the following original publications which are referred to in the text as I–V. The publications are reproduced with kind permission from the publishers.


Author’s contributions

Publication I
Outi Koivistoinen participated in the designing of the experimental work and carried out the laboratory work (construction of the RHA1 expression strain, enzyme assays, purification of the enzyme, northern blot analysis), except the MALDI-TOF MS analysis, analysed and interpreted the results, and collaborated with the other authors to write the article.

Publication II
Outi Koivistoinen participated in the designing and execution of the experimental work (construction of the strains, enzyme assays, purification of the enzymes), except cluster analysis and transcriptional profiling, participated in analysis of the results, and collaborated with the other authors to write the article.

Publication III
Outi Koivistoinen participated in the designing and execution of the experimental work, made strain constructions and enzyme analyses, produced L-xylo-3-hexulose, and performed phenotype analyses, and collaborated with the other authors to write the article.

Publication IV
Outi Koivistoinen participated in the designing of the work, carried out the experimental work including transcription analysis, strain constructions, protein extraction and analysis and HPLC analysis, analysed the results, and collaborated to write the paper together with the other authors.

Publication V
Outi Koivistoinen designed and carried out the toxicity assays of the study and analysed the data, participated in the design and construction of the S. cerevisiae and K. lactis strains, carried out the flask cultivations of the S. cerevisiae and K. lactis strains and analysed the data. Outi Koivistoinen drafted the article and is the corresponding author of the paper.
Contents

Abstract ........................................................................................................... 3
Tiivistelmä ....................................................................................................... 5
Preface ............................................................................................................. 7
Academic dissertation ..................................................................................... 9
List of publications ........................................................................................ 10
Author’s contributions .................................................................................. 11
List of abbreviations ...................................................................................... 14

1. Introduction ............................................................................................. 16
   1.1 Biomass derived sugars .................................................................... 17
      1.1.1 D-Galactose ........................................................................... 18
      1.1.2 L-Rhamnose .......................................................................... 20
   1.2 Catabolism of hexoses ..................................................................... 20
      1.2.1 Embden-Meyerhof-Parnas pathway ........................................ 20
      1.2.2 Entner-Doudoroff pathway ..................................................... 21
      1.2.3 The non-phosphorylative Entner-Doudoroff pathway ............... 23
      1.2.4 D-Galactose catabolism ......................................................... 23
      1.2.5 L-Rhamnose catabolism ......................................................... 25
   1.3 Tricarboxylic acid cycle ..................................................................... 27
   1.4 Glyoxylate cycle ............................................................................... 28
   1.5 Metabolic engineering ....................................................................... 29
   1.6 Examples of biochemicals produced by engineered fungi .......... 30
      1.6.1 Lactic acid ............................................................................. 31
      1.6.2 Citric acid .............................................................................. 33
      1.6.3 Itaconic acid .......................................................................... 34
   1.7 Aims of the study ............................................................................... 35
2. Materials and methods........................................................................................................36

3. Results ................................................................................................................................39
   3.1 L-Rhamnose pathway in S. stipitis .............................................................................39
       3.1.1 Identification of the L-rhamnose 1-dehydrogenase encoding gene ...................40
       3.1.2 Characterisation of the L-rhamnose 1-dehydrogenase ........................................42
       3.1.3 Characterisation of the L-rhamnoate dehydratase ..............................................42
       3.1.4 Characterisation of the L-rhamnose cluster ........................................................43
   3.2 D-Galactose pathway in A. niger ..............................................................................43
       3.2.1 Identification of the galactitol dehydrogenase encoding gene ............................44
       3.2.2 Characterisation of the galactitol dehydrogenase ..............................................45
       3.2.3 Identification of the D-sorbitol dehydrogenase encoding gene ...........................45
       3.2.4 Characterisation of the D-sorbitol dehydrogenase .............................................46
   3.3 Engineering of the glyoxylate cycle for glycolic acid production ..............................47
       3.3.1 Toxicity of glycolic acid ....................................................................................47
       3.3.2 Overexpression of the glyoxylate reductase ......................................................47
       3.3.3 Deletion of the malate synthases ........................................................................48
       3.3.4 Overexpression of the isocitrate lyase and deletion of the cytosolic isocitrate dehydrogenase .............................................48
       3.3.5 D-Glucose repression ....................................................................................49
       3.3.6 K. lactis bioreactor cultivation ............................................................................50

4. Discussion .........................................................................................................................51
   4.1 The fungal L-rhamnose cluster ..............................................................................51
       4.1.1.1 L-Rhamnose dehydrogenase ........................................................................52
       4.1.1.2 L-Rhamnoate dehydratase ............................................................................54
   4.2 The fungal D-galactose pathway ..............................................................................55
       4.2.1 Galactitol dehydrogenase ................................................................................58
       4.2.2 D-Sorbitol dehydrogenase ................................................................................58
   4.3 Biotechnological applications of L-rhamnose and D-galactose ...............................58
   4.4 Glycolic acid production in yeast .............................................................................60
       4.4.1 Benefits and challenges of a fungal host in organic acid production ..................60
       4.4.2 Toxicity of glycolic acid ..................................................................................61
       4.4.3 Engineering of the fungal glyoxylate cycle .......................................................62
       4.4.4 Regulation of the glyoxylate cycle .................................................................64

5. Conclusions and future prospects ..................................................................................66

References .............................................................................................................................68

Appendices
Publications I–V
List of abbreviations

ACO1 aconitase gene
ALD6 cytosolic aldehyde dehydrogenase gene
ATP adenosine triphosphate
bp base pair
CoA coenzyme A
DNA deoxyribonucleic acid
EC Enzyme Commission
ED Entner-Doudoroff
EMP Embden-Meyerhof-Parnas
FAD flavin adenine dinucleotide
gal1 galactokinase gene of *Trichoderma reesei*
GMO genetically modified organism
GLYR1 glyoxylate reductase gene of *Arabidopsis thaliana*
GOR1 glyoxylate reductase gene of *Saccharomyces cerevisiae*
HPLC high performance liquid chromatography
ICL1 isocitrate lyase gene
IDP2 isocitrate dehydrogenase gene
lad1 L-arabitol-4-dehydrogenase gene of *Trichoderma reesei*
ladA L-arabitol-4-dehydrogenase gene of *Aspergillus niger*
larA L-arabinose reductase gene of *Aspergillus niger*
Km Michaelis-Menten constant
KDG 2-keto-3-deoxygluconate
KDGal 2-keto-3-deoxy-galactonate
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>KDPG</td>
<td>2-keto-3-deoxy-6-phosphogluconate</td>
</tr>
<tr>
<td>KDPGal</td>
<td>D-2-keto-3-deoxy-6-phosphate-galactonate</td>
</tr>
<tr>
<td>MLS1</td>
<td>Malate synthase gene</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide, reduced form</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate, reduced form</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>sdhA</td>
<td>D-sorbitol dehydrogenase gene of <em>Aspergillus niger</em></td>
</tr>
<tr>
<td>SGD</td>
<td><em>Saccharomyces</em> Genome Database</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
</tr>
<tr>
<td>Vₘₐₓ</td>
<td>Maximum velocity</td>
</tr>
<tr>
<td>wt</td>
<td>Wild type</td>
</tr>
<tr>
<td>xdhA</td>
<td>NAD(+)‐dependent xylitol dehydrogenase gene of <em>Aspergillus niger</em></td>
</tr>
<tr>
<td>xyrA</td>
<td>D‐xylose reductase gene of <em>Aspergillus niger</em></td>
</tr>
<tr>
<td>ZWF1</td>
<td>D‐glucose‐6‐phosphate dehydrogenase (G6PD) gene</td>
</tr>
</tbody>
</table>
1. Introduction

Depletion of fossil fuel resources and the resulting increase in the prices of oil-based fuels and chemicals have caused a growing interest towards biobased alternatives. Also, environmental challenges such as climate change have increased the demand for green solutions where the end product has more potential of being closer to carbon neutral than the traditional oil based options. Bioethanol is the fuel having the longest history in this field. During the last decade there has been a boom for bioethanol although there has also been severe criticism towards the fuel properties and the apparent greenness of its production. The main concern has been the increasing usage of land area for non-food crop production, which is causing increases in food prices. The vast majority of the biofuels and chemicals currently being produced are derived from corn, sugarcane and other crop species, which could be used for food as well. This is because of their high starch and C6 sugar content, which are easy substrates for industrially relevant microbes such as *Saccharomyces cerevisiae*. A lot of research has been carried out to increase the ability of *S. cerevisiae* and several other yeast species to also utilise C5 sugars such as D-xylose, which are non-food carbon sources that are abundant in many agricultural and forest residues (Hahn-Hägerdal et al. 2007, Matsushika et al. 2009).

For cost effective and environmentally sustainable biofuel and biochemical production, the goal is towards complete substrate utilisation. This process starts from the development of effective pre-treatment processes, which requires the breaking of the highly crystalline and compact structure of cellulose as well as the xylan backbones of hemicellulose. Cellulose and hemicellulose are both abundant in plant cell walls and are very resistant to microbial degradation so a pre-treatment process is needed. The next step involves development of cellulases and hemicellulases, which will further degrade the structures of cellulose and hemicellulose to sugar monomers (Gray et al. 2006).

The growing understanding and increased effectiveness of ethanol production from various starting materials has diversified the end product profile, which now includes many other biobased fuels and chemicals. Some of the biobased chemicals already have a long history such as lactic acid which has mainly been produced microbially by lactic acid bacteria since the 1990s (John et al. 2007). Citric acid is another example of an organic acid naturally produced by a microorganism,
the filamentous fungus *A. niger*. The idea of biobased chemicals is therefore nothing new but the profound understanding of metabolism and the increasing amount of genome sequences and tools for genetic engineering have made the production of biobased fuels and chemicals much more diverse. It is not anymore required that the microbe naturally produces the chemical of interest but completely new pathways can be introduced into the cell. For example, lactic acid is nowadays produced not only by lactic acid bacteria but also several yeast species have been engineered for this purpose and the successful expression of an exogenous lactate dehydrogenase gene has led to strains efficiently producing lactic acid (Sauer et al. 2010). A similar metabolic engineering approach can also be used in the production of several other biochemicals.

1.1 Biomass derived sugars

Plant based biomass consists mainly of polymers: cellulose, hemicellulose, pectin and lignin. The first three are sugar polymers that form a network of cross-linked fibers. Cellulose is a linear polysaccharide consisting of $\beta(1\rightarrow4)$ linked *D*-glucose units and is a structural component of primary and secondary cell walls. Hemicellulose binds pectin to cellulose whereas pectin binds cells together in the middle lamella. Lignin, a group of aromatic polymers, fills the spaces between the other polymers of the cell wall which confers mechanical strength to the structure and prevents its degradation.

The branched structure of hemicellulose and pectin consists of several heteropolymers, containing mainly pentose and hexose monomer units. Hemicelluloses have $\beta$-(1→4)-linked backbones of different sugar molecules in an equatorial configuration but the exact structure of hemicellulose varies depending on the biomass source (for a review on the characteristics of hemicelluloses see Scheller and Ulvskov (2010)). Hemicelluloses include xyloglucans, xylans, mannans, glucomannans, and $\beta$-(1→3,1→4)-glucans. Some of these polysaccharides, for example mannans, are rich in C6 sugars such as *D*-mannose, *D*-galactose, and *D*-glucose. There are also hemicellulose groups such as xylans which are rich in C5 sugars e.g. *D*-xylose and *L*-arabinose. The ratio of these natural polymers and the exact monomer composition varies greatly depending on the source since plant biomass covers everything from algae to hardwood. Some examples of the monomer composition of different plant biomasses are given in Table 1.

Presently, biotechnological applications are only able to efficiently utilise hexose-containing polymers such as cellulose or hemicellulose-derived mannans. Thus far, the focus has been on the utilisation of *D*-glucose which is the preferred substrate for many microbes. There has also been an extensive amount of research carried out to generate microbial strains capable of utilising pentoses. Many of the industrially relevant microbes such as *S. cerevisiae* do not naturally have the catabolic pathways necessary for pentose utilisation. Progress has been made to address this limitation and there are now many *D*-xylose utilising *S. cerevisiae* strains available. However, additional strain engineering is still need-
ed to improve e.g. D-xylose uptake rates and solve redox imbalances before the strains are sufficient for economically feasible industrial applications (Van Vleet and Jeffries 2009).

To date, the utilisation of less abundant sugar monomers such as D-galactose and L-rhamnose has not been well studied and L-rhamnose has been especially neglected. The Leloir pathway for D-galactose catabolism is already well characterised in S. cerevisiae (Sellick et al. 2008) but the alternative fungal D-galactose pathways have not been well studied so far. However, as hemicellulose consists of several different sugar monomers, the efficient utilisation of hemicellulose requires that the less abundant components are also catabolised. Thus, it is important to gain more understanding with respect to the different catabolic pathways of biomass sugars.

1.1.1 D-Galactose

D-Galactose is a monosaccharide differing from D-glucose only in the position of one hydroxyl group on C3. D-Galactose is abundant in nature as a component of hemicelluloses and pectin polysaccharides in plant cell walls.

Softwood is especially rich in galactoglucomannan, which has a backbone of β-(1→4)-linked D-mannose and D-glucose residues with D-galactose side groups. Hardwood also contains galactoglucomannan but glucomannan is the more abundant form. The most abundant hemicellulose polymer in hardwood and cereals is xylan, which is also a source for D-galactose. Xylan is composed of a β-(1→4)-linked D-xylose backbone, which can be substituted by side groups such as L-arabinose, D-galactose, acetyl, ferulic acid ester and D-glucuronic acid residues. D-Galactose is also present in the xyloglucans of cell walls where some of the D-xylose substituents of the β-(1→4)-linked D-glucose backbone can have additional β-D-galactose and α-L-arabinose residues.

As described above, the D-galactose molecule is usually bound to a polysaccharide structure. Thus yeast, filamentous fungi, bacteria, plant cells and animal cells release enzymes such as α-galactosidases, β-galactosidases, and endogalactanases cleaving the substrate into D-galactose monomers, which can be transported into the cells for further utilisation.
Table 1. Biochemical composition of different biomasses as weight percentage of the dry weight in whole material. Gal = D-galactose, Rha = L-rhamnose, Glu = D-glucose, Xyl = D-xylose, Ara = L-arabinose, Man = D-mannose, Fuc = L-fucose. ND = not detected.

<table>
<thead>
<tr>
<th>Biomass</th>
<th>Gal</th>
<th>Rha</th>
<th>Glu</th>
<th>Xyl</th>
<th>Ara</th>
<th>Man</th>
<th>Fuc</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lobolly pine, untreated</td>
<td>4.3</td>
<td>40.9</td>
<td>7.1</td>
<td>10.0</td>
<td></td>
<td></td>
<td></td>
<td>(Kelley et al. 2004)</td>
</tr>
<tr>
<td>Athel, (Tamarix aphylla L), untreated</td>
<td>0.5</td>
<td>49.3</td>
<td>11.8</td>
<td>0.7</td>
<td>0.3</td>
<td></td>
<td></td>
<td>(Zheng et al. 2007)</td>
</tr>
<tr>
<td>Eucalyptus, (Eucalyptus camaldulensis),</td>
<td>2.2</td>
<td>44.5</td>
<td>10.5</td>
<td>0.8</td>
<td>0.3</td>
<td></td>
<td></td>
<td>(Zheng et al. 2007)</td>
</tr>
<tr>
<td>untreated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creeping wild rye, (Leymus triticoides),</td>
<td>0.8</td>
<td>34.0</td>
<td>16.5</td>
<td>3.3</td>
<td>ND</td>
<td></td>
<td></td>
<td>(Zheng et al. 2007)</td>
</tr>
<tr>
<td>untreated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tall wheatgrass, (Agropyron elongatum),</td>
<td>0.7</td>
<td>31.1</td>
<td>16.9</td>
<td>2.8</td>
<td>ND</td>
<td></td>
<td></td>
<td>(Zheng et al. 2007)</td>
</tr>
<tr>
<td>untreated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reed canarygrass, vegetative stage,</td>
<td>1.6</td>
<td>0.1</td>
<td>20.9</td>
<td>11.7</td>
<td>3.0</td>
<td>0.6</td>
<td>0.1</td>
<td>(Bradshaw et al. 2007)</td>
</tr>
<tr>
<td>hydrolysate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reed canarygrass, seed stage, hydrolysate</td>
<td>1.3</td>
<td>0.1</td>
<td>26.5</td>
<td>16.3</td>
<td>2.8</td>
<td>0.6</td>
<td>0.1</td>
<td>(Bradshaw et al. 2007)</td>
</tr>
<tr>
<td>Sugar beet pulp, hydrolysate</td>
<td>5.1</td>
<td>2.4</td>
<td>21.1</td>
<td>1.7</td>
<td>20.9</td>
<td>1.1</td>
<td>0.2</td>
<td>(Micard et al. 1996)</td>
</tr>
<tr>
<td>Corn fibre, hydrolysate</td>
<td>3.6</td>
<td>37.2</td>
<td>17.6</td>
<td>11.3</td>
<td></td>
<td></td>
<td></td>
<td>(Grohmann and Bothast 1997)</td>
</tr>
<tr>
<td>Green seaweed, (Ulva lactuca), hydrolysate</td>
<td>1.0</td>
<td>7.0</td>
<td>8.2</td>
<td>4.5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>(van der Wal et al. 2013)</td>
</tr>
</tbody>
</table>
1. Introduction

1.1.2 L-Rhamnose

L-Rhamnose (L-6-deoxy-mannose) is a C6 deoxy sugar that is enriched in some fractions of plant biomass, such as hemicellulose and pectin. Buckthorn (Rhamnus), poison sumac, and plants in the genus Uncaria are known to have exceptionally high L-rhamnose content. A biosynthetic L-rhamnose pathway in bacteria ensures that the L-rhamnose substrate is abundant enough for use as a building block for their cell walls and capsules (Giraud and Naismith 2000).

Pectin is a structurally complex family of galacturonic acid-rich polysaccharides present in plant cell walls. L-rhamnose is there in the form of rhamnogalacturonan I or rhamnogalacturonan II. The structure of pectin and rhamnogalacturonans is described in more detail in a review by Mohnen (2008). L-rhamnose is sometimes also a component of hemicellulose in the reducing ends of the xylan structure.

Several microorganisms that live on decaying plant material are able to use L-rhamnose as a source of carbon and energy. α-L-rhamnosidase enzymes catalyse a reaction cleaving α-L-rhamnose from natural glycosides containing a terminal α-L-rhamnose. The α-L-rhamnosidase enzyme is widely present in different animal tissues, plants, yeasts, fungi and bacteria (Yadav et al. 2010).

1.2 Catabolism of hexoses

There are several types of D-glucose catabolism routes and these same pathways are also involved in the catabolism of other hexose sugars. The predominant one is the glycolytic route, the Embden-Meyerhof-Parnas pathway. There are also three different types of oxidative routes for hexose catabolism: the Entner-Doudoroff pathway and the non-phosphorylative and semi-phosphorylative version of the Entner-Doudoroff pathway. In addition, also the pentose phosphate pathway can be employed for hexose catabolism. Depending on the substrate and organism only one of the routes or a combination of the routes might be available.

1.2.1 Embden-Meyerhof-Parnas pathway

The Embden-Meyerhof-Parnas (EMP) pathway is the most well known and studied glycolytic pathway where one glucose molecule is converted into two pyruvate molecules in a set of reactions where two ATP and two NADH molecules are formed. In addition to glucose, other hexoses can also be converted to EMP pathway intermediates and thus be similarly converted to pyruvate.

The EMP pathway reactions are anaerobic and the pathway is common among bacteria and eukaryotes. The pathway is not truly an oxidative pathway although it does have an oxidative step when triose phosphate is oxidised to pyruvate. Thus, the term oxidative hexose pathway usually refers to the alternative hexose catabolic routes.

In Archea, the EMP pathway does not usually exist although there are exceptions; a reversible EMP pathway has been found in the hyperthermophilic archeon
1. Introduction

Thermoproteus tenax (Brunner et al. 1998) and a modified EMP pathway in the hyperthermophilic archeon Pyrococcus furiosus (Sakuraba et al. 2004).

1.2.2 Entner-Doudoroff pathway

The Entner-Doudoroff (ED) pathway (Figure 1) is an alternative pathway for glucose catabolism and it is present in many organisms lacking some of the EMP pathway enzymes (e.g. phosphofructokinase). The ED pathway also converts one \( \alpha \)-glucose molecule into two pyruvate molecules but only one molecule of ATP is formed. Instead of the two NADH molecules that the EMP pathway produces, the ED pathway produces a net yield of one NADH and one NADPH molecule. As the net yield of ATP is smaller in the ED pathway than in the EMP pathway, it does not produce energy as efficiently and thus the pathway is usually present under aerobic conditions where the required ATP supply can be maintained by other metabolic pathways.

The first step of the Entner-Doudoroff pathway is an oxidation of glucose-6-phosphate to 6-phosphogluconate, the step being identical to the pentose phosphate pathway. From this point onwards, the ED pathway has only two different steps compared to the EMP pathway or pentose phosphate pathway: 6-phosphogluconate is converted to 2-keto-3-deoxy-6-phosphogluconate (KDPG) by a dehydratase and this reaction is followed by a cleavage of KDPG to glyceraldehyde 3-phosphate and pyruvate. Glyceraldehyde 3-phosphate is then catabolised through the enzymatic steps that are also involved in the EMP pathway.

It has been suggested that the ED pathway predated the EMP pathway and that the EMP pathway has rather been an anabolic pathway than a catabolic pathway from the beginning (Romano and Conway 1996). In any event, the ED pathway is present in all three domains of life although it is most common in gram negative bacteria. The ED pathway was first found in Pseudomonas saccharophila (Entner and Doudoroff 1952) and some years later in Escherichia coli (Eisenberg and Dobrogosz 1967).

As mentioned previously, it is also possible to have different types of sugar catabolism pathways present in the same organism. For example, the archeon Halococcus saccharolyticus uses the EMP type pathway to catabolise \( \alpha \)-glucose whereas \( \alpha \)-fructose is almost exclusively catabolised through the ED pathway (Johnsen et al. 2001).
Figure 1. A schematic figure of the Entner-Doudoroff pathway on the left (yellow), the semi-phosphorylative ED pathway in the middle (blue) and the non-phosphorylative ED pathway on the right (green). The figure has been adapted from Koivistoinen (2008).
1. Introduction

1.2.3 The non-phosphorylative Entner-Doudoroff pathway

Besides the original phosphorylative Entner-Doudoroff (ED) pathway there is a non-phosphorylative and a semi-phosphorylative ED pathway (Figure 1). These pathways are mostly used by Archea but some bacterial species and eukaryotic microbes also have some parts of the pathway. The non-phosphorylative ED pathway was first found in an archaeon *Thermoplasma acidophilum* (Budgen and Danson 1986). The name implies that there is no phosphorylative step: KDG (2-keto-3-deoxygluconate) is cleaved into D-glyceraldehyde and pyruvate by an aldolase. D-Glyceraldehyde is then further converted into D-glycerate by an aldehyde dehydrogenase. Finally, the glycerate kinase converts glycerate to 2-phosphoglycerate in an ATP consuming reaction after which the remaining two steps are identical to the ED pathway. The non-phosphorylative ED pathway yields two pyruvate molecules and only one NADPH molecule and there is no net ATP production.

There is also a semi-phosphorylative form of the pathway (Figure 1) which has been found in some archea and bacteria. The enzymes of the pathway were first identified in *Rhodobacter sphaeroides* by Szymona and Doudoroff (1960). In this pathway, D-glucose is converted to KDG through the same enzymatic conversion steps as the non-phosphorylated pathway. The key difference to the non-phosphorylative pathway is the KDG kinase converting KDG to 2-keto-3-deoxy-6-phosphogluconate (KDPG). The further steps of the pathway have phosphorylative intermediates and are the same as in the phosphorylative ED pathway.

1.2.4 D-Galactose catabolism

Microorganisms use several different pathways for the catabolism of D-galactose. The above presented D-glucose pathways — the EMP, the semi-phosphorylative ED and the non-phosphorylative ED pathway — also play a role in D-galactose catabolism. The most studied is the Leloir pathway, which is present in prokaryotic and eukaryotic microbes as well as in humans (Frey 1996, Holden et al. 2003). For example many yeast species, e.g. *S. cerevisiae*, use the Leloir pathway for D-galactose catabolism. In this pathway D-galactose is converted to D-glucose 1-phosphate, a metabolically more versatile intermediate, which can be further converted to D-glucose 6-phosphate of the EMP pathway by phosphoglucomutase.

In the first step of the Leloir pathway, β-D-galactose is epimerised to α-D-galactose by galactose mutarotase. α-D-galactose is then phosphorylative by an ATP-dependent galactokinase (EC 2.7.1.6, GALK) to D-galactose 1-phosphate. The third enzyme of the pathway is D-galactose 1-phosphate uridylyltransferase (EC 2.7.7.12, GALT) which facilitates transfer of a UMP group from UDP-glucose to D-galactose 1-phosphate generating D-glucose 1-phosphate and UDP-galactose. Finally, recycling of the UDP-galactose to UDP-glucose is catalysed by the UDP-galactose-4-epimerase (EC 5.1.3.2, GALE). The Leloir pathway has attracted a lot of interest as deficiency in the genes encoding the main enzymes of the
1. Introduction

Pathway causes galactosemia, which is a rare but potentially lethal metabolic disorder in humans.

There are also oxidative D-galactose pathways. The De Ley-Doudoroff pathway (De Ley and Doudoroff 1957) that exists in some bacterial species is similar to the semi-phosphorylative ED pathway since the reactions are similar and end products the same: pyruvate and glyceraldehyde-3-phosphate (Figure 2). The first enzyme of the pathway, the D-galactose dehydrogenase, has been characterized from *Pseudomonas saccharophila* (Wangenmayer et al. 1973), *Pseudomonas fluorescens* (Blachnitzky et al. 1974) and partially from *Rhizobium melloti* (Arias and Cerveñansky 1986) and from *Azotobacter vinelandii*. However, in the latter case the substrate specificities are different compared to the other purified D-galactose dehydrogenase enzymes (Wong and Yao 1994). In this pathway, D-galactose is first oxidised to D-galactonic acid-\(\gamma\)-lactone by a NAD-dependent dehydrogenase enzyme followed by a conversion catalysed by D-galactono-\(\gamma\)-lactonase to D-galactonate. D-galactonate is then converted to D-2-keto-3-deoxy-galactonate (KDGal) by a dehydratase. KDGal is then phosphorylated to D-2-keto-3-deoxy-6-phosphate-galactonate by a KDGal kinase and further split to pyruvate and D-glyceraldehyde 3-phosphate by an aldolase.

The non-phosphorylative De Ley-Doudoroff D-galactose pathway is another oxidative D-galactose pathway but the difference in this route to the De Ley-Doudoroff pathway is the lack of phosphorylative intermediates. The non-phosphorylative De Ley-Doudoroff pathway is similar to the non-phosphorylative ED pathway. In this pathway, the steps are identical to the De Ley-Doudoroff pathway up to KDGal. Instead of phosphorylation, KDGal is split into D-glyceraldehyde and pyruvate by an aldolase. This pathway is known to exist in the archaeon *Sulfobus solfataricus* (Lamble et al. 2005) and in *A. niger* (Elshafel and Abdel-Fatah 2001).
1. Introduction

Figure 2. The De Ley-Doudoroff pathway and the non-phosphorylative version of it are oxidative d-galactose catabolism pathways. Up to KDGal the enzymatic steps of the pathways are identical. On the semi-phosphorylative De Ley-Doudoroff pathway (marked by blue) the KDGal is converted to d-2-keto-3-deoxy-6-phosphate-galactonate by a KDGal kinase. On the non-phosphorylative variation of the De Ley-Doudoroff pathway (marked by green) KDGal is split by an aldolase to pyruvate and d-glyceraldehyde.

1.2.5 L-Rhamnose catabolism

There are also two distinctly different pathways known for the catabolism of L-rhamnose: the phosphorylative and the non-phosphorylative. The pathway with
phosphorylative intermediates is found only in bacteria such as E. coli (Power 1967, Wilson and Ajl 1955). All the enzymes and the corresponding genes of the pathway have been described (Moralejo et al. 1993). In this pathway, \( \text{L-rhamnose} \) is first isomerised by \( \text{L-rhamnose isomerase} \) (EC 5.3.1.14) (Wilson and Ajl 1957a, Takagi and Sawada 1964a) and the formed \( \text{L-rhamnulose} \) is phosphorylative by \( \text{rhamnulokinase} \) (EC 2.7.1.5) (Wilson and Ajl 1957b, Takagi and Sawada 1964b) to \( \text{L-rhamnulose 1-phosphate} \), which is further converted to dihydroxyacetone phosphate and \( \text{L-lactaldehyde} \) by \( \text{L-rhamnulose-1-phosphate aldolase} \) (EC 4.1.2.19) (Sawada and Takagi 1964). \( \text{L-lactaldehyde} \) can be then reduced to 1,2-propanediol by \( \text{lactaldehyde reductase} \) (EC 1.1.1.77) or oxidised to lactate by \( \text{lactaldehyde dehydrogenase} \) (EC 1.2.1.22) depending on the redox conditions (Boronat and Aguilar 1979, Boronat and Aguilar 1981, Baldoma and Aguilar 1988). In E. coli, 1,2-propanediol is secreted (Boronat and Aguilar 1979) but in anaerobic conditions, \( \text{Salmonella typhimurium} \) is able to take back the 1,2-propanediol and further convert it to propionate and propanol (Obradors et al. 1988). \( \text{Listeria innocua} \) has a similar \( \text{l-rhamnose catabolism approach} \) in aerobic conditions (Xue et al. 2008).

\( \text{Clostridium phytofermentans} \) was recently found to have similar \( \text{l-rhamnose catabolism} \) where the end products on \( \text{l-rhamnose grown cells} \) were propanol, propionate, ethanol, lactate, and acetate (Petit et al. 2013). Petit et al. also found out that growth of \( \text{C. phytofermentans} \) on \( \text{l-fucose} \) or \( \text{l-rhamnose} \) led to the expression of a variety of lignocellulolytic enzymes such as cellulases, chitinases and mannanases.

The pathway without phosphorylative intermediates has been described in the yeasts \( \text{Aureobasidium (Pullularia) pullulans} \) (Rigo et al. 1985, Rigo et al. 1976), \( \text{Scheffersomyces (Pichia) stipitis} \) (Twerdochlib et al. 1994), \( \text{Debaryomyces polymorphus} \) (Twerdochlib et al. 1994), \( \text{Debaryomyces hansenii} \) (Watanabe et al. 2008) and the bacterium \( \text{Azotobacter vinelandii} \) (Watanabe et al. 2008). The first step of the pathway, \( \text{l-rhamnose dehydrogenase} \) and the corresponding gene \( \text{RhaD} \), have also been found in the archaeon \( \text{Thermoplasma acidophilum} \) (Kim et al. 2012) indicating that the pathway is non-phosphorylative or semi-phosphorylative which is comparable to the non-phosphorylative or semi-phosphorylative ED pathways.

In the fungal non-phosphorylative pathway, \( \text{l-rhamnose} \) is first oxidised to \( \text{l-rhamno-\gamma-lactone} \) by the NAD-utilising \( \text{l-rhamnose 1-dehydrogenase} \) (EC 1.1.1.173). \( \text{l-Rhamno-\gamma-lactone} \) is then hydrolysed to \( \text{l-rhamnose} \) by \( \text{l-rhamno-\gamma-lactonase} \) (EC 3.1.1.65). \( \text{l-rhamnose} \) is converted to 3,6-dideoxy-L-erythro-hexulosonic acid by \( \text{l-rhamnose acid dehydratase} \). 3,6-dideoxy-L-erythro-hexulosonic acid is finally split by an aldolase (\( \text{l-2-keto-3-deoxy-rhamononate aldolase} \)) (EC 4.2.1.-) to pyruvic acid and \( \text{l-lactaldehyde} \) where the latter one can still be oxidised to \( \text{l-lactate} \) in an NAD-coupled reaction by \( \text{l-lactaldehyde dehydrogenase} \) (EC 1.2.1.22). This reaction is the same as in the pathway with the phosphorylative intermediates. However, it has been suggested that the conversion by \( \text{l-rhamno-\gamma-lactonase} \) is not essential for the pathway as lactone can also be formed by spontaneous hydrolysis (Twerdochlib et al. 1994).

There is also a modified version of the non-phosphorylative \( \text{l-rhamnose pathway} \) where the enzyme and corresponding genes have been identified from \( \text{Sphingomonas} \) sp. (Watanabe and Makino 2009). The three first enzymes from...
1. Introduction

The tricarboxylic acid (TCA) cycle (Figure 3), also called the citric acid cycle or the Krebs cycle, provides energy, ATP, for the cell under aerobic conditions and generates NADH which is fed into oxidative phosphorylation. This offers a catabolic route for pyruvate which is formed for example during hexose metabolism. The cycle starts from acetyl-CoA so there is an intermediate step needed between the glycolytic and TCA cycle which requires pyruvate to be converted to acetyl-CoA by the pyruvate dehydrogenase complex. In eukaryotes, the TCA cycle takes place in the mitochondria but as bacteria lack these, the reactions are cytosolic.

In addition to being a direct continuation of hexose and carbohydrate metabolism, the TCA cycle also participates in protein and fat metabolism. These molecules are also first converted into acetyl-CoA which is then fed into the cycle. The net yield of the TCA cycle from one acetyl-CoA molecule is three NADH, two CO₂, one FADH₂ and one GTP molecule.
1. Introduction

1.4 Glyoxylate cycle

The glyoxylate cycle (Figure 3) or glyoxylate shunt is a shortcut of the TCA cycle which allows cells to utilise C2 carbon sources, ethanol and acetate, when complex sources are not available and it further allows the cell to produce carbohydrates from acetyl-CoA. The glyoxylate cycle is present in bacteria, plants, protist and eukaryotic microbes but not in higher eukaryotes.
The glyoxylate cycle shares most of the enzymatic reactions with the TCA cycle and there are only two glyoxylate cycle specific enzymes: malate synthase and isocitrate lyase. The net yield from the glyoxylate cycle is:

\[
2 \text{ acetyl-CoA} + \text{NAD}^+ + 2\text{H}_2\text{O} \rightarrow \text{succinate} + 2\text{CoA} + \text{NADH} + \text{H}^+ 
\]

Except for the isocitrate lyase, the other glyoxylate cycle enzymes are known to have isozymes in *S. cerevisiae*. For citrate synthase, *S. cerevisiae* has three isozymes. The mitochondrial CIT1 is the central enzyme in the TCA cycle, the peroxisomal CIT2 is involved in the glyoxylate cycle and the third mitochondrial citrate synthase, CIT3, also has methylcitrate synthase activity (Kispal and Srere 1991, Lewin et al. 1990, Graybill et al. 2007). For aconitase, *S. cerevisiae* has two distinct genes, ACO1 and ACO2. The Aco1p is found in the mitochondria and in the cytoplasm and it functions both in the TCA cycle and the glyoxylate cycle (Gangloff et al. 1990) while the other isozyme Aco2p is mitochondrial (Vélot et al. 1999). Two different genes with malate synthase activity have been characterised: MLS1 functions in the glyoxylate cycle and DAL7 is part of allantoin catabolism (Hartig et al. 1992). *S. cerevisiae* is known to have three malate dehydrogenase enzymes: Mdh1p is a mitochondrial malate dehydrogenase involved in the TCA cycle (Thompson et al. 1988), Mdh2p is a cytosolic isozyme involved in the glyoxylate cycle and gluconeogenesis during growth on two-carbon compounds (Minard and McAlister-Henn 1991, Gibson and McAlister-Henn 2003) and Mdh3p is a peroxisomal enzyme involved in the glyoxylate cycle as well (Steffan and McAlister-Henn 1992).

### 1.5 Metabolic engineering

The aim of metabolic engineering is to modify existing biological pathways and to design new ones to achieve increased product yields, decreased formation of byproducts and to make cells more suitable for fermentation applications and more tolerant to inhibitors. To achieve this, recombinant DNA technologies are used to engineer the cell. At the beginning of the 1990s, the first articles on metabolic engineering broadened the scope of genetic engineering to cover not only the existing enzyme production processes but to also use similar tools for engineering of entire biochemical pathways leading to metabolite and chemical production (Bailey 1991, Stephanopoulos and Vallino 1991). An important and new idea was also to start combining genetic engineering with modelling for better understanding of the complex metabolic networks.

The metabolic engineering field has evolved tremendously since the early days as the development of molecular biology tools and the growing amount of published genome sequences have made gene deletions and overexpressions a routine process (Raab et al. 2005). Whole pathways including e.g. transporters and regulators along with the pathway enzymes can be engineered and introduced into the host cell. Targeting of the pathway enzymes can be altered to prevent unnecessary transport between different compartments of the cell which can
1. Introduction

lead to reduced flux (Blumhoff et al. 2013). In addition, cofactor specificity of the enzymes can be modified to solve redox imbalances (Nevoigt 2008). Imbalance of cofactors such as insufficient NADPH supply can be an undesired result caused by the introduction of new genes or modification of the existing pathways.

Cell metabolism is a complex system where even small modifications can cause imbalances to the system. Thus, metabolic engineering is a complex process which can lead to undesired outcomes which can negatively affect the viability of the cells. For example, some heterologous genes introduced to the cell can be lethal. In addition, intermediates or products of the new or improved metabolic routes can be toxic and thus prevent high product titres.

Engineered microorganisms are most commonly used for bioprocessing and biocatalysis purposes. There are several examples of industrially relevant products which have been targets of metabolic engineering: ethanol, lactic acid, citric acid, lysine, propanediol, synthetic drug intermediates and therapeutic proteins (Raab et al. 2005). Examples of success stories which have already been commercialised are e.g. the production of lactic acid using engineered yeast (Miller et al. 2011) and the production of artemisinic acid (Paddon et al. 2013), which is a precursor for the malaria drug artemisin.

The traditionally used microbes *E. coli* and *S. cerevisiae* are still widely used but as genome sequences and engineering tools are already available for a variety of microbes other microbes have also attracted a lot of interest during the past decade. High growth and fermentation rates, tolerance to the end product and performance robustness are important characteristics for the host organism. For example, low pH tolerance is a desired trait for organic acid production. *E. coli* does not tolerate low pH and thus many yeast species have been preferred hosts in organic acid production.

1.6 Examples of biochemicals produced by engineered fungi

Although there are various examples of chemicals produced by engineered or non-engineered microbes, the vast majority of chemicals are still produced from petrochemical resources by chemical synthesis. In many cases, product titres, production rates and yields are still far below the necessary levels needed for economic industrial production of biochemicals. Even though sustainability is a key issue and gaining more and more interest, the production should also be economically feasible. This generally means, especially for bulk chemicals, that the product titres should be high, substrates cheap, fermentation processes straightforward and the purification process simple and inexpensive.

At the moment, there are only a few examples of biotechnically produced bulk chemicals such as lactic acid, citric acid and itaconic acid which will be shortly reviewed in the next chapters to give examples of biochemicals already produced on an industrial scale. These are all produced efficiently by yeast and fungi. Lactic acid is produced by engineered yeast species. Citric acid is produced by *A. niger*, which is its natural producer and high titres are obtained solely by optimising the
growth conditions. Itaconic acid is naturally produced by another Aspergillus species, Aspergillus terreus. However, in all of these examples, strain engineering has either been essential or at least led to improved production and offered a possibility to more efficiently use different substrates.

1.6.1 Lactic acid

The chemical synthesis of lactic acid was developed in the 1960s (Wee et al. 2006). In the 1990s, the fermentation methods of lactic acid bacteria that naturally produce lactic acid began to be more economically feasible (Lasprilla et al. 2012). Very rapidly, fermentation became the predominant production method with 90% of the lactic acid being produced by fermentation in the United States by the late 1990s (Budhavaram and Fan 2009). Nowadays the percentage is approximately the same worldwide (Lasprilla et al. 2012). According to Castillo et al. 2013, the production of lactic acid was expected to reach 259,000 metric tons by the year 2012.

Lactic acid bacteria are a clade of gram-positive bacteria including the genera Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetracoccus, Vagococcus, and Weissella (Stiles and Holzapfel 1997). The clear benefit of microbial production is the intrinsic nature of many microbes to produce optically pure L(+) or D(-) lactic acid whereas chemical synthesis always results in a racemic mixture of lactic acid. Optical purity is crucial for high molecular weight polylactic acid (PLA) polymerisation. In addition to being an important chemical for the production of pharmaceuticals, cosmetics, foods and detergents, significant amounts of lactic acid is also used in PLA production to produce biodegradable plastics.

Production of lactic acid by microbial fermentation is a highly optimised process, where the fermentation capacity is high and the final lactic acid concentrations are over 100 g l\(^{-1}\) (Hofvendahl and Hahn-Hägerdal 2000). For industrial production of lactic acid the genus Lactobacillus is most commonly used since they are homofermentative and produce high yields of lactic acid (Auras et al. 2004). Although fermentation is a superior production method when compared to the chemical synthesis route, there are still some drawbacks in the use of lactic acid bacteria. In general, lactic acid bacteria require a complex growth media which includes vitamins and amino acids. In addition, the pH needs to be maintained between pH 5-7 for optimal production of lactic acid, which requires the use of excessive amounts of neutralisation agents. The pKa of lactic acid is 3.78 meaning that for pHs below this value, lactic acid is mainly in its undissociated form which also makes the purification process easier and cheaper (Sauer et al. 2010). To allow for economically feasible production, it is a real challenge to keep the production costs low enough when producing a bulk chemical such as lactic acid where the acceptable manufacturing cost has been estimated to be under 0.8 USD per kg (Wee et al. 2006).
1. Introduction

Figure 4. The pathways and the most central enzymes needed for the production of lactic acid, citric acid and itaconic acid.

Yeast do not naturally produce lactic acid as they lack the lactic acid dehydrogenase needed to convert pyruvate into lactic acid (Figure 4) and tend to produce ethanol instead. However, engineered yeast strains can be very effective lactic acid producers. In addition to the required introduction of the lactic acid dehydrogenase gene, production can be increased by other modifications such as the deletion of pyruvate decarboxylase, PDC, to prevent undesired ethanol production (Porro et al. 1999, Ishida et al. 2006, Osawa et al. 2009, Ikushima et al. 2009). The benefit of yeast is their tolerance to low pH conditions compared to lactic acid bacteria and they also require less expensive growth media. Because of these economic benefits, the production of lactic acid in yeast has been of interest for almost 20 years (Sauer et al. 2010) and the efficient production of lactic acid has been shown in many different yeast species e.g. in *S. cerevisiae* (Ishida et al. 2006), *Candida utilis* (Ikushima et al. 2009), *Candida boidinii* (Osawa et al. 2009), *S. stipitis* (Ilmen et al. 2007) and *K. lactis* (Porro et al. 1999). The potential of yeast
as a low pH tolerant lactic acid producer has already been exploited for several years by industry (Vink et al. 2010, Miller et al. 2011).

1.6.2 Citric acid

Citric acid is an intermediate product of the TCA cycle (Figure 4) and the glyoxylate cycle. Citric acid is a natural preservative that is used as an acidifier, and as a flavouring and chelating agent in the food industry. Already in 1917, James Currie published an article on *A. niger* citric acid production (Currie 1917). Before that, *A. niger* had been considered mainly as an oxalic acid producer but Currie’s studies showed that citric acid was produced in large quantities. He also studied different media compositions and found out that inorganic salts were essential for citric acid production. Shortly after Currie’s findings, in 1919, the first industrial production process using *A. niger* began in Belgium (Papagianni 2007). *A. niger* is still the production host used in industrial processes but the strains have been improved during the years by mutagenesis and selections and the product titres, production yields and rates have been improved by optimising the media and cultivation conditions (Kirimura et al. 2011). Submerged fermentation (mainly batch but also fed-batch and continuous fermentation), surface fermentation and solid state fermentation processes are all used in industrial production. The yield of citric acid is often more than 70 % of the theoretical yield of the carbon source (Papagianni 2007).

Although the history of citric acid production in *A. niger* is long, there is still work carried out to find ways to optimise the production. A mathematical model of *A. niger* metabolism revealed that there are at least 13 enzymes involved whose activity should be modified in order to achieve an optimal citric acid production strain (Alvarez-Vasquez et al. 2000). These include proteins of transport processes, of the TCA cycle, of glycolysis and of CO₂ fixation in oxaloacetate formation. In the study it was claimed that by affecting the concentrations of the selected enzymes, the production rate of citric acid could be increased by more than 12-fold. This shows that even though the organism was already a naturally good citric acid producer there are still multiple ways to optimise the product titres, product yields and rates. However, relatively little has been done so far in order to engineer *A. niger* strains for enhanced citric acid production, which is thought to be attributed to the fact that the organism is relatively challenging to engineer. De Jongh and Nielsen (2008) have tested TCA cycle related modifications; malate dehydrogenase, *MDH2*, and cytosolic soluble fumarate reductase, *FRDS1*, from *S. cerevisiae* and two cytosolic targeted fumarases *FUM1S* and *FUMRS* from *S. cerevisiae* and *Rhizopus oryzae*, respectively, and found out that they increased *A. niger* citric acid production when compared to the wild type strain.

Citric acid production has also been studied in other eukaryotic microbes. The yeast *Yarrowia lipolytica* has been found to be a promising citric acid producer and it has been thought to have several advantages compared to *A. niger*. *Y. lipolytica* has a broader substrate range, a decreased sensitivity to low dissolved oxygen
concentrations and heavy metals and higher citric acid product yields (Moeller et al. 2012). The drawback of using yeast as a production host instead of \textit{A. niger} is the production of the byproduct isocitric acid. However, this problem is possible to be solved by metabolic engineering. The invertase-encoding \textit{SUC2} gene of \textit{S. cerevisiae} and multiple copies of the endogenous \textit{ICL1} isocitrate lyase encoding gene increased the citric acid production and reduced isocitric acid production in \textit{Y. lipolytica} (Kruse et al. 2004, Förster et al. 2007a, Förster et al. 2007b). In addition, deletion of the ATP-citrate lyase gene, \textit{ACL1}, catalysing transformation of citric acid into oxaloacetate and \textit{CH}_{3}COS-CoA was found to be beneficial for citric acid production in \textit{Y. lipolytica} (Liu et al. 2013).

There is a growing interest to use biomass waste and by-products as cheap starting materials for citric acid production. A by-product of bio-diesel production, glycerol, has been examined in a number of studies and so far the highest titre reported in glycerol based media using unmodified \textit{Y. lipolytica} has been 112 g l\(^{-1}\) of citric acid (Rymowicz et al. 2010). In another example, 68.3 g l\(^{-1}\) of citric acid was produced from Jerusalem artichoke tuber hydrolysate as a starting material when using an engineered \textit{Y. lipolytica} strain where the ATP-citrate lyase, \textit{ACL1} encoding gene was deleted and the \textit{ICL1} encoding gene was overexpressed (Wang et al. 2013). In a study using date waste as a substrate, 98.4 g l\(^{-1}\) of citric acid was produced by an unmodified \textit{A. niger} strain (Acourene and Ammouche 2012), and apple pomace resulted in 312.32 g of citric acid per kg of dry solids through solid state fermentation using wild type \textit{A. niger} (Dhillon et al. 2013).

In addition to \textit{Y. lipolytica}, other yeast species, mainly from the genus \textit{Candida}, have been studied as potential candidates for citric acid production. An unmodified \textit{Candida oleophila} strain produced 74.2 g l\(^{-1}\) of citric acid by optimising the nitrogen feed at constant C/N ratio, which was found to be essential for efficient citric acid production (Anastassiadis et al. 2005). A comparison of five different \textit{Candida} species showed that \textit{Candida guilliermondii} was the most efficient citric acid producer when using glycerol as a substrate with yields similar to that of \textit{Y. lipolytica} (West 2013).

1.6.3 Itaconic acid

Itaconic acid is one among the twelve chemicals listed on the \textit{Top value added chemicals from biomass} report published by the USA Department of Energy in 2004 (Werpy and Petersen 2004). Thus, there has been significant interest in finding efficient ways to produce this chemical, which can be used as a building block of different polymers. For example, it can be used as a copolymer with acrylic acid and in styrene-butadiene systems.

Itaconic acid is naturally produced by \textit{A. terreus} but the challenge remains its relatively low production levels, as the titres do not exceed 80 g l\(^{-1}\) (Willike and Vorlop 2001). Citric acid is an intermediate of the metabolic pathway leading to itaconic acid production but two additional enzymes, aconitase, \textit{aco1}, and \textit{cis}-aconitate decarboxylase, cad, are needed for the synthesis of itaconic acid (Figure 4).
1. Introduction

Still, the titres of citric acid produced in industrial processes remains around three-fold higher when compared to the production of itaconic acid. This difference is partially explained by the different localisation of these enzymes. Citric acid is produced in the mitochondria and in A. niger and A. terreus, the aconitate is also located mainly in the mitochondria (Jaklitsch et al. 1991a, Jaklitsch et al. 1991b) whereas the cis-aconitate decarboxylase of A. terreus is cytosolic (Jaklitsch et al. 1991b). Thus, transport between different compartments of the cell is needed. Two different approaches have been used to solve this particular problem. Over-expression of a putative mitochondrial carrier, mttA, or a putative di-carboxylate carrier, mfsA, which are both located in the same gene cluster as cadA of A. terreus, resulted in an increase in itaconic acid production in A. niger (Li et al. 2013). The alternative approach did not focus on transport but the effect of targeting expression of the cad gene of A. terreus to the mitochondria of A. niger. This approach was also found to increase itaconic acid production (Blumhoff et al. 2013). However, in both of these studies, the production of itaconic acid remained low (i.e. below 2 g l⁻¹).

1.7 Aims of the study

Plant based biomasses such as hemicellulose and pectin are gaining interest as the development of biochemicals and biofuels is increasingly moving towards the use of lignocellulosic biomass and other residual plant waste materials instead of the use of glucose rich crop species such as corn and sugarcane, which compete with the food production. Thus, it is important to understand the catabolism of the different sugar monomers such as D-galactose and L-rhamnose, which were targets of this study. It is also important to understand the diversity of metabolic routes involved in hexose catabolism in addition to the prevailing Embden-Meyerhof-Parnas glycolytic pathway as this will contribute to the general understanding of fungal physiology and makes further studies possible on evolution of fungal species and gives targets for genetic modifications.

For successful and economically feasible production of biochemicals and biofuels, it is often essential to engineer the fungi in order to make it an efficient production host. The TCA cycle is involved in the production of several organic acids which are already on the market or at least getting close to the production levels where industrial production starts to be a realistic aim. The glyoxylate cycle is a bypass route of the TCA cycle sharing many of its enzymes but being differentially regulated. So far, an engineered glyoxylate cycle has not been studied in fungal organic acid production but it offers a possibility to produce the useful chemical glycolic acid. Glycolic acid can be used in cosmetics and as a precursor for poly-glycolic acid, which has excellent gas-barrier properties and is thus a desired material for the packing industry. In this study, the aim was to demonstrate glycolic acid production in yeast by engineering the glyoxylate cycle.
2. Materials and methods

The yeast and mould species used in this work are generally well characterised and their genome sequences are publicly available: for *Saccharomyces cerevisiae* at the Saccharomyces Genome Database [http://www.yeastgenome.org/](http://www.yeastgenome.org/), for *Kluyveromyces lactis* at the Genolevures database [http://genolevures.org/klla.html](http://genolevures.org/klla.html) and *Scheffersomyces (Pichia) stipitis* and *Aspergillus niger* at the Joint Genome Institute website [http://genome.jgi-psf.org/Picst3/Picst3.home.html](http://genome.jgi-psf.org/Picst3/Picst3.home.html) and [http://genome.jgi-psf.org/Aspni5/Aspni5.home.html](http://genome.jgi-psf.org/Aspni5/Aspni5.home.html), respectively. The parent strains used in this thesis are listed in Table 2.

Table 2. Yeast and filamentous fungus strains used in this work.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Genotype</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. stipitis</em></td>
<td>CBS 6054</td>
<td>wt</td>
<td>I, II</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>pyrG null mutant of CBS113.46 (ATCC 1015)</td>
<td>pyrG</td>
<td>III, IV</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>CEN.PK2-1D</td>
<td>MATa ura3-52; trp1-289; leu2-3,112; his3Δ 1; MAL2-8°C; SUC2</td>
<td>I–V</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>H3675, a xylose utilising mutant of CEN.PK113-1A²</td>
<td>MATa his3 leu2 trp1 ura3::XYL1-XYL2 xks1::XKS1 MAL2-8°C SUC2</td>
<td>V</td>
</tr>
<tr>
<td><em>K. lactis</em></td>
<td>H3954 / CBS 2359³</td>
<td>MATa ura3 leu2 his3:: loxP ku80::loxP</td>
<td>V</td>
</tr>
</tbody>
</table>

¹ (Toivari et al. 2001, Jouhten et al. 2008)
² (de Jong-Gubbels et al. 1998)
³ (Heinisch et al. 2010)
2. Materials and methods

The most essential methods for each publication are listed in Table 3. The main focus of the work in Publications I, III and IV was the characterisation of new genes coding for enzymes involved in the catabolic pathways. Thus, protein purification was involved in all these publications and it was also performed in Publication I. Enzyme activity measurements were then used to characterise the enzymes.

In Publication I, the candidate for the encoding gene was found using MALDI-TOF peptide identification. Once the encoding gene had been identified, Northern hybridization was used to confirm that the gene was indeed transcribed in the cells which were grown on L-rhamnose containing medium.

In addition to protein purification and enzyme characterization, Publication II also involved expression array and transcriptome profiling to identify the genes which are part of the L-rhamnose cluster and to further analyse the fungal species where the cluster or some of its genes were present.

Publications III and IV focused on D-galactose catabolism, more specifically to the identification of two genes involved in the pathway. In these publications, qPCR and transcription analyses were used to identify the genes which are up-regulated in growth conditions where D-galactose or some of its degradation products are present. Metabolite analysis was finally used to confirm that the characterised enzymes produced the presumed metabolites.

Publication V focused on the metabolic engineering of the glyoxylate cycle in the yeasts S. cerevisiae and K. lactis. This involved expression of a heterologous gene in yeast and the modification of the pathway by overexpressions and deletions of the relevant endogenous genes. Cultivations and metabolite analysis were then used to compare the glycolic acid production of the different strains. Enzyme activity measurements were also used in this publication to analyse the effect of the deletion of a regulatory gene on the enzyme activity of a selected glyoxylate cycle enzyme.

More detailed description of the materials and methods used in the Publications I–V are described in each publication.
### Table 3. Methods used in Publications I–V.

<table>
<thead>
<tr>
<th>Method</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td>Comparative analysis of gene clustering</td>
<td>II</td>
</tr>
<tr>
<td>Constructing a fungal deletion strain</td>
<td>III–V</td>
</tr>
<tr>
<td>Enzyme activity measurements</td>
<td>I–V</td>
</tr>
<tr>
<td>Expressing heterologous genes in yeasts</td>
<td>V</td>
</tr>
<tr>
<td>Expression array and transcriptome profiling</td>
<td>II</td>
</tr>
<tr>
<td><em>K. lactis</em> fed-batch bioreactor cultivation</td>
<td>V</td>
</tr>
<tr>
<td>Metabolite analysis; HPLC, CE or NMR</td>
<td>III–V</td>
</tr>
<tr>
<td>Northern hybridisation</td>
<td>I</td>
</tr>
<tr>
<td>Protein purification and MALDI-TOF peptide identification</td>
<td>I</td>
</tr>
<tr>
<td>Protein purification (HIS-tag) and characterisation</td>
<td>II, III, IV</td>
</tr>
<tr>
<td>qPCR and transcription analysis</td>
<td>III, IV</td>
</tr>
<tr>
<td><em>S. stipitis</em> bioreactor cultivations</td>
<td>II</td>
</tr>
<tr>
<td>Shake flask cultivations</td>
<td>V</td>
</tr>
</tbody>
</table>
3. Results

The fungal *S. stipitis* L-rhamnose pathway was studied in the two first Publications (I and II). The gene coding for the L-rhamnose 1-dehydrogenase gene (*RHA1*) was identified and the corresponding enzyme (Rha1) characterised and the L-rhamnoate dehydratase enzyme (Lra3) was characterised. In addition, the gene cluster for L-rhamnose catabolism was characterised and the distribution of its genes studied in the kingdom of Fungi.

In the study of D-galactose (Publications III and IV), the fungal *A. niger* D-galactose pathway was studied. The galactitol dehydrogenase (*ladB*) and D-sorbitol dehydrogenase (*sdhA*) genes were identified and the corresponding enzymes (LadB and SdhA) characterised.

The overall understanding of cell metabolism was applied in the study (Publication V) where an engineered glyoxylate cycle was used in the production of glycolic acid. *S. cerevisiae* was used as a model organism to test different modifications. However, the product titres in *S. cerevisiae* were not very high, only around 1 g l\(^{-1}\) in flask cultivations even after several genetic modifications. Another yeast host, *K. lactis*, was then tested in order to see if the host selection would be an important factor in glycolic acid production. The most important modifications were carried out in *K. lactis*, which was producing over two-fold the amount of glycolic acid in flask cultivations compared to *S. cerevisiae*. Based on this promising result, *K. lactis* was chosen for fed-batch bioreactor cultivation in order to demonstrate glycolic acid production.

3.1 L-Rhamnose pathway in *S. stipitis*

The enzymes of the non-phosphorylative L-rhamnose pathway had been described before but none of the corresponding genes had been identified prior to the work done in Publication I, where the gene coding for the first enzyme of the fungal oxido-reductive L-rhamnose pathway, NAD\(^+\) dependent L-rhamnose 1-dehydrogenase, was identified. Characterisation of the L-rhamnose cluster was finalised in the Publication II by studying upregulation and distribution of the genes that are part of the cluster. In addition, the third enzyme of the pathway, L-rhamnoate dehydratase, was expressed in a heterologous host *S. cerevisiae* to analyse its function and its enzyme kinetics. The catabolic L-rhamnose pathway and the genes involved are presented in Figure 5.
3. Results

3.1.1 Identification of the \(\text{L-rhamnose 1-dehydrogenase}\) encoding gene

The first gene of the non-phosphorylative \(\text{L-rhamnose}\) pathway, \(\text{L-rhamnose 1-dehydrogenase}\), was identified based on its \(\text{L-rhamnose 1-dehydrogenase}\) enzyme activity. The \(\text{L-rhamnose 1-dehydrogenase}\) enzyme activity of the purified cell extract of \(\text{S. stipitis}\) grown on \(\text{L-rhamnose}\) media was detected based on the NAD\(^+\) consumption of the enzyme. The active fractions separated by native PAGE were zymogram stained and the visible bands were then eluted and applied to an SDS-PAGE gel where four differently sized bands were revealed. The proteins had estimated sizes of 30, 35, 52 and 70 kDa (Publication I, Figure 2). The 30 kDa band was the most promising candidate based on the comparison of the SDS-PAGE analysis containing the active fractions from the zymogram stained gel and the SDS-PAGE analysis of the active fractions after the DEAE column purification, which revealed that a 30 kDa protein was visible only in the first mentioned case. The protein was trypsinated and the peptide masses of each fragment were analysed by MALDI-TOF after which the protein was identified based on the BLASTp search over the \(\text{S. stipitis}\) genome sequence. The masses 555.247, 900.475, 1199.639, 1761.782, 1872.708 and 2552.586 matched with a putative \(\text{D-glucose 1-dehydrogenase}\), which had 258 amino acids and a calculated molecular mass of 27.102 Da and which was hence renamed as \(\text{L-rhamnose 1-dehydrogenase, Rha1}\). Rha1 belongs to the protein family of short-chain alcohol dehydrogenases.

To confirm the role of the \(\text{L-rhamnose 1-dehydrogenase}\) in \(\text{L-rhamnose}\) catabolism of \(\text{S. stipitis}\), northern analysis was carried out with different carbon sources; \(\text{L-rhamnose, D-glucose, maltose, D-galactose, D-xylose}\) and a glycerol-ethanol mixture. The result clearly showed that the gene was upregulated only on \(\text{L-rhamnose}\) and thus the study showed that \(\text{L-rhamnose 1-dehydrogenase}\) is active when \(\text{L-rhamnose}\) is present as the carbon source.
Figure 5. The three different pathways for L-rhamnose catabolism. The uppermost pathway (blue arrows) has been identified in several bacteria and it has phosphorylative intermediates. The pathway in the middle (green arrows) is the non-phosphorylative L-rhamnose pathway, which has been identified in fungi and some bacteria. The lowermost pathway (pink arrows) is the variation of non-phosphorylative pathway found from Sphingomonas sp.
3. Results

3.1.2 Characterisation of the \( \text{L-rhamnose 1-dehydrogenase} \)

In order to determine the kinetic properties of the Rha1 enzyme, the corresponding gene was expressed with both C- or N-terminal 6xHIS-tags in \( S. \text{cerevisiae} \) from a multicopy vector under control of a constitutive promoter. In \( S. \text{stipitis} \), the CTG codon is translated to serine and not to leucine as usually so this codon was changed into TCG in order to get the same amino acid sequence for the protein expressed in \( S. \text{cerevisiae} \). Unfortunately, the activity of both of the tagged proteins was significantly reduced or they did not exhibit any activity towards \( \text{L-rhamnose} \). Hence, the gene was expressed in \( S. \text{cerevisiae} \) without additional tags and the enzyme activity studies were performed with crude extract since the tag could have possibly interfered with catalytic activity and falsified the kinetic properties of the enzyme. The enzyme used NAD\(^+\) as a cofactor; the \( V_{\text{max}} \) was 200 ± 20 nkat mg\(^{-1}\) protein, and the \( K_m \) was 0.2 ± 0.03 mM (Publication I, Figure 3). The highest reaction rate and affinity for a substrate were detected towards \( \text{L-rhamnose} \) with the \( V_{\text{max}} \) being about 200 ± 20 nkat mg\(^{-1}\) protein and the \( K_m \) was 1.5 ± 0.025 mM. Activity was observed also towards \( \text{L-lyxose} \) and \( \text{L-mannose} \) with the \( V_{\text{max}} \) being 170 ± 20 nkat mg\(^{-1}\) protein and 75 ± 10 nkat mg\(^{-1}\) protein and the \( K_m \) being 5 ± 0.5 mM and 25 ± 5 mM, respectively (Publication I, Figure 3). The reverse activity with NADH and \( \text{L-rhamnoate} \) was not observed. The activity of the enzyme was found out to be pH dependent and the highest activity was detected at pH 9.5 (240 ± 25 nkat mg\(^{-1}\) protein). At pH 6.8, the activity was less than half of the maximum (100 ± 10 nkat mg\(^{-1}\) protein) and at pH 8.0, the activity was 200 ± 20 nkat mg\(^{-1}\) protein.

3.1.3 Characterisation of the \( \text{L-rhamnoate dehydratase} \)

Although the gene coding for the fungal \( \text{L-rhamnoate dehydratase} \), \( \text{LRA3} \), had already been identified by Watanabe et al. (2008) by the time of the Publication II, the biochemical characterisation was still not completed as their attempts to express the corresponding gene from \( D. \text{hansenii} \) in \( E. \text{coli} \) were unsuccessful. In order to finalise the characterisation of the enzymes acting on the \( \text{L-rhamnose} \) pathway, the \( \text{LRA3} \) gene from \( S. \text{stipitis} \) was expressed in \( S. \text{cerevisiae} \) from a multicopy vector under a constitutive promoter. The gene had two CUG codons, which were replaced by UCG codon in order to also get the same desired amino acid sequence in \( S. \text{cerevisiae} \). As already mentioned, \( S. \text{stipitis} \) has a diverging codon usage where CUG codes for serine and not leucine as in many other organisms. Both N- and C-terminal 6xHIS tags were added to the gene but the activity was either lost (C-terminal) or reduced 95 % (N-terminal) so further characterisation was done using crude extracts. The enzyme had quite a low affinity towards \( \text{L-rhamnonate} \) with the \( K_m \) being 25 mM and a reaction rate \( V_{\text{max}} \) of 0.11 nkat mg\(^{-1}\) protein (Publication II, Figure 3). Other sugar acids including \( \text{L-mannoate} \), \( \text{L-lyxone} \), \( \text{L-gulonate} \), \( \text{D-galactonate} \), \( \text{L-ga lactonate} \), \( \text{D-fuconate} \), \( \text{D-ribonate} \), \( \text{L-arabonate} \), \( \text{D-xylonate} \) and meso-galactarate were also tested but activity was only observed towards
3. Results

L-mannoate and L-lyxonate. L-Mannoate activity was similar to that of L-rhamnate but L-lyxonate activity was reduced 95%.

3.1.4 Characterisation of the L-rhamnose cluster

Analysis of 32 fungal genomes revealed that the L-rhamnose cluster was present in Basidiomycota and Ascomycota genuses (Publication II, Table 1). The full cluster was determined to include RHA1, LRA2, LRA3, LRA4, the genes encoding for enzymes of the oxidative L-rhamnose pathway, and a putative transcription factor TRC1, which was always adjacent to the cluster. The transcription factor had Zn(II)2Cys6 (IPR001138) and IPR007219 domains typical of fungal transcription factors and it was always located close to RHA1 and LRA3. As these three genes were found to be in close proximity to each other’s they were specified to constitute the core cluster.

The full L-rhamnose cluster was conserved only in close relatives of S. stipitis, which include C. guilliermondii, Candida lusitaniae and D. hansenii (Publication II, Figure 2). According to the literature, all of these species are also able to grow on L-rhamnose. There were also two Basidimycota species, Ustilago maydis and Cryptococcus neoformans, which have been reported to grow on L-rhamnose. C. neoformans has the core cluster but U. maydis lacks the core cluster and only has the RHA1 gene, thus this species is likely to have an alternative pathway for L-rhamnose catabolism.

Transcriptome analysis showed that all of the L-rhamnose pathway genes including L-lactaldehyde dehydrogenase, LADH, and the transcription factor TRC1, were upregulated on L-rhamnose. Another putative transcription factor, FST14, and a group of genes encoding for enzymes related to metabolism (ICL1, PCK1, FBP1, CAT2, YAT1, ALD6, FAA24, BLG5, HIB1, and GLR2) as well as three transporters (HXT4, FUC1 and SFC1) were also induced on L-rhamnose (Publication II, Figure 4). Unfortunately, the efforts to delete the transcription factors TRC1 and FST14 were not successful and thus their role in L-rhamnose catabolism is based only on genome and transcriptome analysis.

3.2 D-Galactose pathway in A. niger

The gene coding for the first enzyme of the D-galactose oxido-reductive pathway in A. niger had already been identified and the Publication III continued the study by identifying the gene coding for the NAD+ dependent galactitol dehydrogenase enzyme responsible for converting galactitol into L-xylo-3-hexulose. The oxido-reductive L-arabinose and D-galactose pathways have similarities and in Trichoderma reesei, the enzyme converting L-arabitol to L-xylulose, L-arabitol 4-dehydrogenase (Lad1), also converts galactitol to L-xylo-3-hexulose. This T. reesei gene was found to be upregulated in the presence of D-galactose and galactitol in addition to the previously known fact that it is upregulated on L-arabinose (Richard et al. 2001). However, this was not the case in A. niger, where the gene ladA, homologous to
3. Results

*T. reesei lad1* was induced only on L-arabinose but not on D-galactose or galactitol. This study showed that *A. niger* had a separate galactitol dehydrogenase enzyme, *ladB*, catalysing the conversion of galactitol to L-xylol-3-hexulose.

The characterisation of the oxido-reductive D-galactose pathway in *A. niger* was continued in Publication IV by identifying the NAD+ dependent D-sorbitol dehydrogenase gene, *sdhA*, which codes for the enzyme converting D-sorbitol to D-fructose. The gene was upregulated on D-galactose, galactitol and D-sorbitol indicating that it is involved in both, D-galactose and D-sorbitol catabolic pathways.

### 3.2.1 Identification of the galactitol dehydrogenase encoding gene

In order to identify the gene coding for the galactitol dehydrogenase, induction of several genes homologous to the *A. niger* L-arabitol 4-dehydrogenase (*ladA*) was tested on D-galactose. A close homologue, the JGI183900 (An16g01710), named as *ladB* was found to be upregulated when grown on D-galactose and galactitol but not on L-arabinose or D-glucose (Publication III, Figure 2), which made it a potential candidate for the gene coding for galactitol dehydrogenase.

The *ladB* gene was deleted from the *A. niger* strain ATCC 1015 and the null mutant strain was compared to the wild type strain and to a strain where *xyrA*, the D-xylose dehydrogenase encoding gene, had been deleted. Since *A. niger* is unable to grow on D-galactose as the sole carbon source, the growth was tested in the presence of 2 % D-galactose and 0.025 % D-xylose. The addition of D-xylose was so small that it alone was not enough to promote growth but high enough to activate the transcription of *xyrA*, which encodes D-xylose reductase, the enzyme also required for D-galactose reduction. On this carbon source mixture, both deletion strains grew poorly compared to the growth of the wild type strain (Publication III, Figure 3). The growth of the *ladB* null mutant strain was then also tested on 2 % galactitol. The growth was completely abolished in the mutant strain as the wild type control grew normally (Publication III, Figure 3).

As the growth on D-galactose was only reduced but not completely arrested, transcription studies were performed to find out whether other genes related to the L-arabinose or to the putative D-galactose Leloir pathway were involved in *A. niger* D-galactose metabolism. The expression of *A. niger larA* (L-arabinose reductase), *xyrA*, *ladA*, *ladB*, and three putative Leloir pathway genes (Filiphi et al. 2009), galactokinase (*gal1*), galactose 1-phosphate uridylyltransferase (*gal7*), and UDP-glucose 4-epimerase (*gal10*), were studied in the cells grown on 2 % D-galactose and 0.025 % D-xylose. Of these genes, only *xyrA* and *ladB* were found to be upregulated (Publication III, Figure 3). The *ladB* null mutant strain was also tested and found to have increased *xyrA* expression levels compared to the wild type strains with obviously no expression of the deleted *ladB* gene. These results combined with the mutant phenotype studies showed that *ladB* does indeed have a significant role in D-galactose and galactitol metabolism and there are no additional genes carrying out the same function.
3. Results

3.2.2 Characterisation of the galactitol dehydrogenase

To further evaluate the function and difference of LadB and LadA, the encoding genes were expressed with a C-terminal 6xHis tag in the *S. cerevisiae* CEN.PK2-1D strain from a multicopy vector under control of a constitutive promoter. After the purification, the activities of these two different His-tagged proteins were tested in the presence of different polyols. The results were fairly similar with the main difference being the affinity and reaction rate for L-arabitol (Publication III, Figure 4), as expected. LadA had a low $K_m$ for L-arabitol, 1 mM, and a low $V_{max}$ of 6.17 nkat mg$^{-1}$. Instead, the corresponding values for LadB were much higher: 55 mM and 24.17 nkat mg$^{-1}$, respectively. The affinity of the two enzymes to galactitol was comparable with the $K_m$ being around 200 mM in both cases. Of the other tested polyols, significant activities were also detected with ribitol and xylitol and to a lesser extent with D-sorbitol, but no activity was observed with mannitol and D-arabitol.

The characterisation of LadB was finalised by testing the product of the enzyme reaction using HPLC and NMR studies. Although it was already shown that LadB is responsible for galactitol conversion as a part of D-galactose metabolism in *A. niger*, it was not yet proved that the product is indeed L-xylo-3-hexulose. L-xylo-3-hexulose was produced in vitro by purified *T. reesei* Lad1 enzyme using galactitol as the substrate and then correspondingly by *A. niger* LadB enzyme and galactitol. In both cases, the product was then identified and purified by HPLC and the structure confirmed by NMR. These results showed that in both cases, L-xylo-3-hexulose was produced and thus it was confirmed that *A. niger* galactitol dehydrogenase converts galactitol to L-xylo-3-hexulose.

3.2.3 Identification of the D-sorbitol dehydrogenase encoding gene

To continue the identification of the enzymes and corresponding genes of the D-galactose oxidoreductive pathway in *A. niger*, it was hypothesised that the last step could be a conversion of D-sorbitol to D-fructose. There has been an earlier study in *Aspergillus nidulans* suggesting that the final step would actually precede via L-sorbose (Fekete et al. 2004) but since there are no known enzymes synthesising L-sorbose from L-xylo-3-hexulose this was not seeming to be the most probable option. In *A. niger*, the activity of sorbitol dehydrogenase and fructokinase have been shown to be induced on D-sorbitol (Desai et al. 1967) but the corresponding genes are not known. Taking into account this prior information, transcription analysis of a selection of putative genes coding for medium chain dehydrogenases was done. As xylitol dehydrogenases are known to have overlapping functions with D-sorbitol dehydrogenases, transcription of the xylitol dehydrogenase, *xdhA*, was also analysed. However, the result clearly excluded this option as *xdhA* was upregulated only on L-arabinose but not on D-sorbitol, galactitol or D-galactose. Of the other genes tested, An08g09380 (JGI37988), An05g02260 (JGI212968), An03g05190 (JGI50731), An14g03510 (JGI185262), and An09g03900 (JGI188914) showed no upregulation on L-arabinose, D-galactose, galactitol or
D-sorbitol. Instead, JGI53356 (An07g01290) was strongly upregulated on sorbitol and was also induced by galactitol (Publication IV, Figure 2) making it the most promising candidate for being the D-sorbitol dehydrogenase encoding gene and thus it was named as sdhA.

To show the relationship of sdhA to D-galactose metabolism, the gene was deleted and the sdhA deletion strain was compared to the wild type strain and to the ladB null mutant strain. The sorbitol dehydrogenase activities of these three different strains, which prior to the enzyme activity measurement had been induced on galactitol, showed that the sdhA deletion strain had almost no sorbitol dehydrogenase activity and even the ladB deletion strain had significantly decreased activity levels compared to the wild type strain. The observation that even the ladB deletion had a major effect on D-sorbitol dehydrogenase activity supports the hypothesis that the SdhA enzyme is involved in D-galactose metabolism.

3.2.4 Characterisation of the D-sorbitol dehydrogenase

In order to characterise the SdhA, it was expressed with a C-terminal 6xHIS-tag in the S. cerevisiae CEN.PK2-1D strain from a multicopy vector under control of a constitutive promoter. The purified protein was shown to have activity on D-sorbitol, xylitol and L-iditol (Publication IV, Figure 4), but not with other tested polyols such as mannitol, ribitol, galactitol, L-arabitol, or D-arabitol. NAD$^+$ was used as a cofactor in the reaction. The $V_{\text{max}}$ and $K_m$ values for D-sorbitol, xylitol and L-iditol were quite similar: $1330 \pm 170$ nkat mg$^{-1}$ and $50 \pm 5$ mM for D-sorbitol, $1420 \pm 170$ nkat mg$^{-1}$ and $50 \pm 3$ mM for xylitol and $1500 \pm 85$ nkat mg$^{-1}$ and $65 \pm 10$ mM for L-iditol. In the reverse reaction NADH was used as a cofactor and the enzyme was active with D-fructose and L-sorbose.

As the reverse enzymatic reaction was also able to use L-sorbose as a substrate, HPLC was used to confirm that the product of the D-sorbitol dehydrogenase catalysed reaction was indeed D-fructose and not L-sorbose. The purified enzyme was incubated with NADH in combination with either D-fructose or L-sorbose and with NAD$^+$ in combination with D-sorbitol. In the reverse reaction with D-fructose, sorbitol was identified as a reaction product. Since the HPLC peaks of the sorbitol and fructose were partially overlapping, the forward direction of the reaction was also tested with D-sorbitol and NAD$^+$. This reaction resulted in fructose formation (Publication IV, Figure 5). In the reaction with L-sorbose, iditol was produced. L- and D-forms of the compounds cannot be distinguished by HPLC. However, oxidation of D-sorbitol can result in formation of only L-sorbose or D-fructose so it was shown that the A. niger D-sorbitol dehydrogenase is converting D-sorbitol to D-fructose as proposed by the hypothesis.
3.3 Engineering of the glyoxylate cycle for glycolic acid production

Glycolic acid production was studied in two different yeast species in Publication V. Expression of the heterologous Arabidopsis thaliana glyoxylate reductase GLYR1 gene in the yeasts S. cerevisiae and K. lactis resulted in glycolic acid production. The production was further increased by directing the flux more efficiently towards glyoxylate by deleting the malate synthase (MLST) and isocitrate dehydrogenase (IDP2) genes. In S. cerevisiae, the gene encoding for isocitrate lyase (ICL1) was also overexpressed and the glucose repression of the glyoxylate cycle was reduced by deleting the regulatory subunit of the type 1 protein phosphatase Glc7p gene (REG1). Finally the production of glycolic acid was demonstrated by flask cultivations and by fed-batch bioreactor cultivation with the engineered K. lactis production strain.

3.3.1 Toxicity of glycolic acid

Neither of the tested yeast species, S. cerevisiae nor K. lactis produced detectable amounts of glycolic acid under normal growth conditions. To test if yeast was a suitable production host for glycolic acid production, its tolerance to glycolic acid was tested. Toxicity studies with different glycolic acid concentrations between 0 and 50 g l⁻¹ at pH 3 and at pH 5 were carried out. The pKa of glycolic acid is 3.83 and thus at pH 3 glycolic acid is mainly in the undissociated form and at pH 5 in its dissociated form. Optical density (OD₆₀₀) measurements reflecting the growth of the cells showed that at pH 5 both yeast species tolerated glycolic acid quite well and K. lactis was able to grow almost normally still when the glycolic acid concentration was 30 g l⁻¹ (Publication V, Figure 2). For S. cerevisiae, 30 g l⁻¹ of glycolic acid already caused reduced growth and both yeast species had significantly reduced growth curves at a concentration of 50 g l⁻¹. At pH 3, glycolic acid was more toxic with growth inhibition already starting at a concentration of 10 g l⁻¹ in K. lactis. In S. cerevisiae, the effect was not so significant at the lower pH with growth only being clearly inhibited at a concentration 30 g l⁻¹. At a glycolic acid concentration of 50 g l⁻¹, there was no growth observed in either of the species.

3.3.2 Overexpression of the glyoxylate reductase

Affinities for different substrates of glyoxylate reductases described in the literature were compared. A. thaliana GLYR1, having the highest described affinity for glyoxylate was chosen and the encoding gene was custom synthesised as a codon optimised version for S. cerevisiae. The endogenous S. cerevisiae glyoxylate reductase, GOR1, was also overexpressed in a multicopy plasmid under a constitutive promoter. However, this did not lead to glycolic acid production. Only the overexpression of GLYR1 resulted in some glycolic acid production but the glycolic
3. Results

Acid production was only just above the detection limit, around 0.1 g l\(^{-1}\) (Figure 6). All further strain constructions were carried out with GLYR1.

3.3.3 Deletion of the malate synthases

Malate synthase, \(\text{MLS1}\), is a glyoxylate cycle specific gene converting glyoxylate into malate. In glycolic acid production, the aim is to convert glyoxylate into glycolic acid and thus the conversion to malate is undesirable. To prevent malate formation, the \(\text{MLS1}\) gene was deleted. To ensure that the reaction was not replaced by another malate synthase enzyme, the \(\text{DAL7}\) gene coding for a malate synthase acting on the allantoin pathway in \(\text{S. cerevisiae}\), was also deleted. The flask cultivation results showed that production of glycolic acid was increased in the \(\text{mls1}\) null mutant strain but the additional \(\text{dal7}\) deletion did not further increase the product titre (Figure 6). Deletion of the \(\text{MLS1}\) gene was also carried out in \(\text{K. lactis}\). The confirmation of a successful deletion was done by enzyme assay, yeast colony-PCR and testing growth on media having ethanol as carbon source as the disrupted glyoxylate cycle causes a no growth phenotype on C2 carbon sources.

3.3.4 Overexpression of the isocitrate lyase and deletion of the cytosolic isocitrate dehydrogenase

In \(\text{S. cerevisiae}\), the deletion of the cytosolic isocitrate dehydrogenase was done in combination with the overexpression of the endogenous isocitrate lyase gene. In addition to the malate synthase, \(\text{Mls1p}\), isocitrate lyase is the other glyoxylate cycle specific enzyme. It splits isocitrate to glyoxylate and succinate, a step which is important in glycolic acid production since the next step involves the conversion of glyoxylate to glycolic acid by glyoxylate reductase.

Isocitrate dehydrogenase, \(\text{IDP2}\), was deleted from both species, \(\text{S. cerevisiae}\) and \(\text{K. lactis}\), in order to more efficiently direct the flux towards glycolic acid by preventing the conversion of isocitrate to \(\alpha\)-ketoglutarate. Compared to the earlier \(\text{S. cerevisiae mls1}\) null mutant strain where \(\text{GLYR1}\) was overexpressed, the overexpression of \(\text{ICL1}\) and deletion of \(\text{IDP2}\) almost doubled the glycolic acid production (Figure 6). In \(\text{K. lactis}\), isocitrate lyase overexpression was not carried out but this host was still a clearly better glycolic acid producer than \(\text{S. cerevisiae}\).
3. Results

Figure 6. Glycolic acid production in shake flask cultivations after 96 hours for different engineered *S. cerevisiae* and *K. lactis* strains grown on media containing a mixture of 2 % ethanol and 2 % D-xylose as carbon sources. The highest titres were achieved in the *K. lactis* glycolic acid production strain. Error bars represent ± the standard error of the mean (SEM; n = 3).

3.3.5 D-Glucose repression

To allow for a broad use of different substrates for glycolic acid production, it would be important to be able to utilise different sugars and convert them to glycolic acid. The challenge here is glucose repression of the glyoxylate cycle. The flask cultivation results showed that the best titres are achieved only in the presence of ethanol (Publication V, Figure 3). Some glycolic acid is also produced from D-glucose but it can be assumed that the yeast first converts glucose to ethanol and only then to glycolic acid. In order to overcome the glucose repression, the regulatory subunit of type 1 protein phosphatase Glc7p gene, *REG1*, was deleted from the *S. cerevisiae* glycolic acid production strain (pGLYR1, mls1-Δ1, dal7-Δ1, idp2-Δ1::ICL1). However, for this strain there was no observable effect on glycolic acid production regardless of the substrate used.

To further study the effect of *REG1*, isocitrate lyase enzyme assays were also carried out and the *reg1* null mutant strain was compared to the non-modified CEN.PK113-1A based parent strain and to the strain where the isocitrate dehy-
3. Results

drogenase IDP2 had been deleted and replaced by isocitrate lyase overexpression. In all of the three strains, the isocitrate lyase activity was clearly the highest in cells grown on ethanol-glycerol media. However, on glucose grown cells, there were some differences and the reg1 null mutant strain showed higher isocitrate lyase activity than the parent strain and the strain where the enzyme was overexpressed. Still the activity was only about one fourth of the activity measured from the cells grown on ethanol-glycerol media. The isocitrate lyase activity was also measured from D-xylose grown cells but there were almost no differences observed between the three different strains (Publication V, Figure 4). The deletion of REG1 clearly had an effect on isocitrate lyase activity of D-glucose grown cells but as the enzyme activity was still much higher in ethanol-glycerol grown cells and as the deletion did not have a clear effect on glycolic acid production it is obvious that there are also other regulators which have an important effect on the glyoxylate cycle enzymes.

3.3.6 *K. lactis* bioreactor cultivation

Flask cultivations are a relatively quick and easy method for studying the effects of different engineering approaches but they only offer a limited amount of information and the growth conditions cannot be properly followed. Bioreactor cultivations on the other hand offer the possibility to feed the strain with the substrate, usually resulting in better product titres being achieved. The *K. lactis* glycolic acid production strain (MATa ura3 leu2 his3::loxP ku80::loxP mls1Δ1, idp2Δ1, pGLYR1) was cultivated in an aerobic fed-batch bioreactor cultivation at pH 5 on media containing D-xylose and ethanol as carbon sources. The concentration of D-xylose was kept above 5 g l⁻¹ and ethanol between 1 and 5 g l⁻¹ and the situation was monitored with on-line HPLC.

At the beginning of the bioreactor cultivation there was a 75 hour batch phase, in which 6.7 g l⁻¹ of glycolic acid was produced. After this, feeding was started and continued for 115 hours to get a final titre of 14.8 g l⁻¹ of glycolic acid (Publication V, Figure 6). Small amounts of malate and acetate were detected as by-products in the process. Glycolic acid yields were calculated at the time points of 95.5 hours and 167.5 hours and they were higher during the first 95.5 hours and then started to decrease at the end of the bioreactor cultivation. At 95.5 hours, the glycolic acid yield from ethanol was 0.52 g g⁻¹ and from ethanol and D-xylose 0.44 g l⁻¹ and at 167.5 hours 0.32 g g⁻¹ and 0.28 g g⁻¹, respectively. The theoretical maximum yield from ethanol was calculated to be 1.00 C-mol C-mol⁻¹, from D-xylose 0.67 C-mol C-mol⁻¹ and from D-glucose and glycerol 0.67 C-mol C-mol⁻¹. The yield from ethanol at 95.5 hours in this *K. lactis* cultivation was around one third of the theoretical yield. More detailed information on yields and specific accumulation rates of the substrates and main products are presented in the Publication V.
4. Discussion

The fungal oxido-reductive L-rhamnose and D-galactose pathways are discussed below separately. The discussion on the fungal L-rhamnose pathway focuses on the L-rhamnose cluster and especially its two enzymes, L-rhamnose 1-dehydrogenase and L-rhamnose dehydratase. The discussion on the fungal D-galactose pathway mainly focuses on galactitol dehydrogenase and D-sorbitol dehydrogenase. In addition, the pathway and its similarities and differences to the alternative fungal oxido-reductive D-galactose pathways and L-arabinose catabolism is discussed in detail. Finally, different biotechnical applications are considered where this knowledge can be applied.

The last part of the discussion relates to glyoxylate cycle engineering and its challenges with respect to glucose repression. The main focus concerns glycolic acid production.

4.1 The fungal L-rhamnose cluster

L-Rhamnose studies were carried out in the yeast *S. stipitis*, which is best known for its superior capacity for xylose fermentation (van Dijken et al. 1986) and it is closely related to passalid yeasts found in the gut of wood ingesting beetles (Suh et al. 2003). *S. stipitis* is able to utilise a wide range of different sugars with L-rhamnose being one example and this combined with the fact that the genome sequence of the organism is known (Jeffries et al. 2007) made it a suitable organism to study L-rhamnose catabolism.

The fungal oxido-reductive L-rhamnose pathway with non-phosphorylative intermediates had been described in *A. pullulans*, *D. hansenii* and in *S. stipitis*. However, before this study had started, none of the genes coding for the enzymes of the pathways had been characterised. The L-rhamnose 1-dehydrogenase encoding gene, *RHA1*, was the first enzyme of the pathway that was identified in Publication I. Shortly after that, Watanabe et al. (2008) identified the other enzyme encoding genes from *S. stipitis* and *D. hansenii*: *LRA2* coding for L-rhamnono-y-lactonase, *LRA3* coding for L-rhamnonate dehydratase and *LRA4* coding for L-2-keto-3-deoxyrhamnonate aldolase. All of these genes were expressed in *E. coli* (Watanabe et al. 2008) but the expression of L-rhamnolactone dehydrogenase was unsuccessful and thus it was heterologously expressed in *S. cerevisiae* in Publication II. Watanabe et al.
4. Discussion

(2008) also found the L-rhamnose cluster in S. stipitis and in several other fungi and bacteria. Most of the enzymes involved in the pathway of S. stipitis, D. hansenii and Azobacter vinelandii were also characterised. However, a careful cluster analysis was not done in this study so Publication II provided a more thorough understanding of the evolution of the cluster and also specified the full cluster consisting of RHA1, LRA2, LRA3, LRA4 and TRC1 genes and the core cluster consisting of RHA1, LRA3 and TRC1 genes. In addition, two transcription factors, TRC1 and FST14, were induced on L-rhamnose. TRC1 was always adjacent to the cluster and thus it was also found to be part of L-rhamnose cluster and the core cluster.

The results showed that the full L-rhamnose cluster was conserved only in close relatives of S. stipitis, which include C. guilliermondii, C. lusitaniae and D. hansenii. All of these species are also able to grow on L-rhamnose. In addition, two Basidomycota species, U. maydis and C. neoformans have been reported to grow on L-rhamnose although they both lack the core cluster. This suggests that an alternative pathway also exists for L-rhamnose catabolism but the cluster studied in this Publication I is part of the L-rhamnose catabolism found in close relatives of S. stipitis. As the attempts to delete the L-rhamnose 1-dehydrogenase encoding gene from S. stipitis were unsuccessful, it is not known if alternative L-rhamnose pathways are also present in this species.

4.1.1.1 L-Rhamnose dehydrogenase

L-Rhamnose dehydrogenase is a sugar dehydrogenase which catalyses the oxidation of a sugar to a sugar acid. The first L-rhamnose dehydrogenase enzyme was already characterised in A. pullulans in the 1970s (Rigo et al. 1976) and a few years later it was found out that the enzyme is D-glucose and D-galactose repressed (Vieira et al. 1979). Later a similar catabolite repression was reported for the S. stipitis L-rhamnose dehydrogenase (Twerdochlib et al. 1994) and this is supported also by the findings of this study.

In addition to L-rhamnose, L-rhamnose dehydrogenase was found to have activity on L-lyxose and L-mannose. This is different than for the enzyme characterised in A. pullulans as this enzyme was not active on these two sugars (Rigo et al. 1976). The S. stipitis L-rhamnose dehydrogenase seems to be less specific and this is also supported by the affinity of the A. pullulans enzyme being higher towards L-rhamnose. The \( K_m \) of A. pullulans L-rhamnose dehydrogenase is 0.2 mM (Rigo et al. 1976) as the S. stipitis enzyme in this study had a \( K_m \) of 1.5 mM. L-rhamnose, L-lyxose and L-mannose all share identical configuration from C1 to C4 as illustrated in the Figure 4 of Publication I. For the case of D-ribose, where the hydroxyl group of C4 was in the opposite configuration, there was no activity detected. The differences in enzyme promiscuity between S. stipitis and A. pullulans indicate that in S. stipitis C1–C4 stereochemical configuration is essential for recognition by the L-rhamnose 1-dehydrogenase whereas the A. pullulans enzyme likely requires the entire C1-C6 configuration for recognition. In addition, the attachments of the C6, such as the hydroxyl group of L-mannose, inhibit the
recognition by the *A. pullulans* enzyme. However, as L-lyxose and L-mannose are very rare sugars in nature and there is no need for their catabolism, this characteristic probably does not indicate that the L-lyxose and L-mannose catabolism would have evolved differently in these two fungal species.

L-Rhamnose 1-dehydrogenase belongs to the short chain dehydrogenases and it has a glycine-rich T-G-X₃-G-X-G motif and a YXXXK active site (Figure 7), which are typical for classical short chain dehydrogenases (Kavanagh et al. 2008). The T-G-X₃-G-X-G motif functions as a coenzyme binding region and it is important for the maintenance of the central β-sheet. Short chain dehydrogenases are a large group of oxidoreductases that are grouped by function as the sequences of the group are heterogeneous. In addition to being present in carbohydrate metabolism, short chain dehydrogenases include enzymes involved in lipid, amino acid, cofactor, hormone and xenobiotic metabolism (Kavanagh et al. 2008).

In eukaryotic microbes, there are still not many enzymes characterised which convert sugar to sugar acid and even fewer of their encoding genes are known. In *S. cerevisiae*, there are two D-arabinose dehydrogenases, *ARA1* (Kim et al. 1998) and *ARA2* (Amako et al. 2006); the first being NADP⁺ dependent and the latter being NAD⁺ dependent. Both of these enzymes convert D-arabinose into erythroascorbic acid and the proteins belong to the family of aldo-keto reductases. In *T. reesei*, a NADP⁺ dependent D-xylose dehydrogenase converting D-xylose into D-xylonate-γ-lactonase belongs to the GFO-IDH-MocA oxidoreductase protein family (Berghäll et al. 2007). In *Aspergillus oryzae*, the first D-glucose dehydrogenase was already characterised in 1937 (Ogura and Nagahisa 1937) and in 1967, a *A. oryzae* D-glucose dehydrogenase was found out to be FAD dependent (Bak 1967). More recently, the D-glucose dehydrogenase was also characterised from *A. niger* (Müller 1977) and *A. terreus* (Tsujimura et al. 2006). These enzymes convert D-glucose to D-gluconic acid or to D-glucono-δ-lactone. Mori et al. (2011) screened *Aspergillus* FAD dependent D-glucose dehydrogenases from genomic databases and found one from *Aspergillus flavus* and one from *A. niger* having high specificity and relatively high activity towards D-glucose. The genes were subsequently characterised as FAD dependent D-glucose dehydrogenases. D-Glucose dehydrogenases and D-glucose oxidases are used in blood glucose monitoring systems. Although many of the enzymes have been characterised, the corresponding genes of the fungi derived FAD dependent D-glucose dehydrogenases are not known.

There are also a few other short chain dehydrogenases where the enzyme has been characterised but the encoding gene is not known. It is also unknown into which protein family these enzymes belong to. These enzymes include a NADP⁺ dependent D-glucose dehydrogenase from *Schizosaccharomyces pombe* (Tsai et al. 1995), a NAD⁺ dependent D-galactose dehydrogenase converting D-galactose into D-galactonic acid-γ-lactone from *A. niger* (Elshafei and Abdel-Fatah 2001), and a NADP⁺ dependent D-xylene dehydrogenase from *Pichia quercuum* which converts D-xylene into D-xylonic acid (Suzuki and Onishi 1973).
4. Discussion

Figure 7. The S. stipitis Rha1p where the TGX_3GXG and YX_3K motifs typical to short chain dehydrogenases have been marked.

4.1.1.2 L-Rhamnose dehydratase

L-Rhamnose dehydratase is a sugar-acid dehydratase converting L-rhamnose to 3,6-dideoxy-L-erythro-hexulosonic acid, which is a synonym to L-2-keto-3-deoxyrhamnonic acid. The gene encoding the L-rhamnose dehydratase in S. stipitis, LRA3, was identified by Watanabe et al. (2008) but the heterologous expression of the gene in E. coli was unsuccessful. In this study, the gene was heterologously expressed in S. cerevisiae. Sometimes, expression of eukaryotic genes in prokaryotes and vice versa is challenging since the differences in gene expression and post-translational modifications can lead to the production of non-functional proteins. LRA3 expression with a HIS-tag was also unsuccessful in S. cerevisiae. Although the size of the tag is small it may sometimes interfere with protein activity (Terpe 2003). Often this can be solved by changing the terminus of the tag but Lra3 remained inactive regardless of the placement of the tag. The other cause for the inactive protein might be the imidazole used for elution of HIS-tagged protein from the Ni\(^{2+}\)-NTA matrix. Although imidazole is commonly used, it is also known to result in protein aggregates and thus can cause an inactive enzyme (Terpe 2003).

Additional tagging systems or elution materials were not tested but expression without additional tags resulted in an active enzyme, which had activity towards L-rhamnate, L-mannoate and to a lesser extent, L-lyxonate. The substrate specificity is similar to the substrate specificity of bacterial L-rhamnose dehydratases characterised in A. vinelandii (Watanabe et al. 2008), E. coli (Rakus et al. 2008) and Sphingomonas sp. (Watanabe and Makino 2009). In addition to these three sugar acids, the A. vinelandii enzyme had minor activity towards L-fuconate, D-mannoate, and D-gluconate. The E. coli enzyme had a minor activity towards D-gulonate but it was so low that they were not able to determine the $K_m$ value. All these bacterial enzymes were expressed in E. coli with an N-terminal 6x-HIS tag making the inactivation problem only specific to the S. stipitis enzyme.

The S. stipitis LRA3 was determined to be part of the enolase superfamily, where all of the above mentioned bacterial L-rhamnose dehydrogenases also
belong. Enolases are a mechanistically diverse group of enzymes which require Mg$^{2+}$ (Gerlt et al. 2005).

4.2 The fungal D-galactose pathway

*A. niger* is a filamentous fungus able to grow on a variety of different carbon sources and it is the cause of the “black mould” found on fruits and vegetables and grows widely in nature on different types of decaying biomass. D-Galactose catabolism was studied in the citric-acid producing *A. niger* strain ATCC 1015, from which the genome sequence has been published (Andersen et al. 2011).

*A. niger* has genes homologous to the genes of the Leloir pathway (Flipphi et al. 2009) but the transcription study of D-galactose grown cells in Publication III did not show any upregulation of these genes. Elshafei and Abdel-Fatah (2001) described a non-phophorylative De Ley-Doudoroff pathway in *A. niger*. Although the enzyme activities were described, the corresponding genes have not been identified. A less explored route is the third D-galactose pathway, the oxido-reductive pathway, which is the subject of this thesis and was studied in *A. niger* in Publications III and IV. Shortly after their publication, the missing link of the pathway, the gene coding for L-xylo-3-hexulose reductase, was also identified (Mojzita et al. 2012).

Prior to this study, the pathway had been partly identified in *T. reesei* and *A. nidulans*, where it was shown to be similar to the L-arabinose pathway. The differences of the enzymes acting on the *T. reesei* and *A. niger* pathways are presented in Figure 8. As shown partially in this study, in *A. niger*, all the enzymes acting on D-galactose and L-arabinose catabolism are pathway specific but this is not the case for *T. reesei*. In *T. reesei*, the first enzyme of the pathway, D-xylose/L-arabinose reductase, Xyl1, is responsible for both D-galactose and L-arabinose metabolism (Seiboth et al. 2007). In addition, the second enzyme of the pathway, L-arabitol dehydrogenase, Lad1, is shared with the L-arabinose and D-galactose pathway in *T. reesei* (Pail et al. 2004). This enzyme can also partially compensate for the xyitol dehydrogenase enzyme, Xdh1 (Seiboth et al. 2003). Only the third step of the pathways have distinct enzymes in *T. reesei* where L-xyululose reductase, Lxr3, acts on the L-arabinose pathway converting L-xyululose to xyitol (Metz et al. 2013) whereas in the D-galactose pathway, the Lxr4 enzyme converts L-xylo-3-hexulose to D-sorbitol (Mojzita et al. 2012). Then again, the last step of the *T. reesei* pathway has the same enzyme, xyitol dehydrogenase Xdh1, which catalyses the conversion of xyitol to D-xylulose in the L-arabinose pathway and conversion of D-sorbitol to D-fructose in the D-galactose pathway as well as the conversion of xyitol to D-xylulose in D-xylulose catabolism (Seiboth et al. 2003).

*T. reesei* is known to have the Leloir pathway with the oxido-reductive pathway operating simultaneously, which was shown by Seiboth et al. (2004). In that study, the deletion of the galactose kinase encoding gene, *galI*, did not result in growth arrest on D-galactose but the simultaneous deletion of galactokinase and the L-arabinitol-4-dehydrogenase encoding gene, *ladI*, did. However, this study showed that this is not the case in *A. niger*. *A. niger* has also a Leloir pathway and
4. Discussion

an oxido-reductive pathway for D-galactose catabolism but not all enzymes for the oxido-reductive D-galactose and L-arabinose catabolism are shared. A. niger was also shown to have one aldose reductase, xyrA, for D-galactose and D-xylose conversion and the other one, larA, for L-arabinose catabolism (Mojzita et al. 2010).

In A. nidulans it has been suggested that galactitol of the oxido-reductive D-galactose pathway is converted to L-sorbose by an unidentified enzyme and then further to D-sorbitol (Fekete et al. 2004). However, in Publication III it was shown that a different route is used in A. niger and T. reesei since the intermediate is L-xylo-3-hexulose and not L-sorbose.

In addition to the enzymes acting on the pathway, the regulation of the D-galactose catabolism differs between different Aspergillus species. In A. nidulans, GalX and GalR are responsible for the regulation of D-galactose catabolism with the first one being part of the oxido-reductive pathway and the latter one being part of the Leloir pathway (Christensen et al. 2011). In A. niger, only GalX is present and similarly to A. nidulans it has been found to regulate the oxido-reductive D-galactose pathway but it is not involved in the regulation of Leloir pathway genes (Gruben et al. 2012).

It was observed in Publication III that the A. niger strain ATCC 1015 was not able to grow on D-galactose when the oxido-reductive pathway was blocked. When combining this finding with the result showing that Leloir pathway genes were not upregulated in the presence of D-galactose, it supports the hypothesis that the oxido-reductive pathway is the major pathway for D-galactose utilisation in A. niger.

Although A. niger has all the enzymes of the oxido-reductive D-galactose pathway, it cannot grow on media containing D-galactose as the sole carbon source (de Vries et al. 2005, Meijer et al. 2011). However, addition of a small amount of D-xylose which is less than what is sufficient to support growth itself, did allow the fungi to grow. This observation is supported by a study by Fekete et al. 2012 where it was suggested that transport of D-galactose in A. niger is growth stage dependent. The conidiospores lack the ability to transport D-galactose but it is present in the mycelia (Fekete et al. 2012). Thus, some other carbon source is needed at the beginning of the growth phase. This is not a problem for A. niger in natural growth environments where D-galactose is never the only carbon source available but is always accompanied by D-xylose.

The D-galactose transporters have not been identified in A. niger and the detailed mechanism of the growth stage regulation of D-galactose uptake is not known. However, a similar type of growth stage dependent regulation has been identified in some of the transporters of T. reesei (Metz et al. 2011) and A. nidulans (Tazebay et al. 1997, Amillis et al. 2004, Pantazopoulou et al. 2007) and thus, this kind of growth stage dependent transport behaviour is not very rare.
Figure 8. The oxido-reductive D-galactose and L-arabinose pathways in A. niger and T. reesei. The genes coding for the enzymes involved in A. niger pathways are in blue and the genes involved in the T. reesei pathway are in black. In T. reesei the first enzyme of the pathway, D-xylose reductase, Xyl1, is involved in the catabolism of three sugars, D-galactose, L-arabinose and D-xylose. Whereas in A. niger, there is a distinct sugar specific aldose reductase involved in each of the pathways. For the second step, the dehydrogenase enzyme Lad1 in T. reesei is the same for galactitol and L-arabitol. In addition, the xylitol dehydrogenase, Xdh1 converting xylitol to D-xylulose and D-sorbitol to D-fructose is the same in all three pathways in T. reesei catabolism. For A. niger, there are distinct enzymes for each step of all three different pathways. The figure has been adapted from Mojzita et al. 2012.
4. Discussion

4.2.1 Galactitol dehydrogenase

A. niger galactitol dehydrogenase, ladB, converting galactitol into L-xylo-3-hexulose is an enzyme specific to the D-galactose pathway. Unlike the T. reesei lad1, which is functional in D-galactose and L-arabinose pathways. A. niger ladB is upregulated only on galactitol and D-galactose and not on L-arabinose as shown in Publication III. In addition, the growth on D-galactitol was also reduced in a ladB null mutant strain. L-Xylo-3-hexulose was found to be the product of the LadB catalysing reaction. This was confirmed with HPLC and NMR studies to ensure that the product was indeed L-xylo-3-hexulose and not L-sorbose as has been suggested for A. nidulans.

4.2.2 D-Sorbitol dehydrogenase

D-sorbitol induced sorbitol dehydrogenase activity had already been detected in A. niger in 1967 (Desai et al. 1967) but the corresponding gene had not been identified until this study. D-Sorbitol dehydrogenase belongs to a family of medium-chain dehydrogenases. It is the last enzyme of the oxido-reductive D-galactose pathway. Overall, medium-chain alcohol dehydrogenases, especially D-sorbitol dehydrogenases share basic structural and functional similarity with xylitol dehydrogenases (Lunzer et al. 1998). In T. reesei, it has been suggested that the xylitol dehydrogenase Xdh1 also carries out the oxidation of D-sorbitol (Seiboth and Metz 2011). However, this was not the case in the A. niger D-galactose pathway as the transcription study showed that the A. niger xylitol dehydrogenase, xdhA, is not upregulated on D-sorbitol but there is a separate D-sorbitol dehydrogenase, which catalyses the conversion of D-sorbitol into D-fructose and is upregulated on D-sorbitol and also had a delayed and reduced upregulation on galactitol.

In a recent publication, Gruben et al. (2012) suggested that in A. niger another gene, putative dehydrogenase deh1, might be similar to sdhA and also involved in D-galactose catabolism. The gene was found to be induced on D-galactose. In addition, they found a putative reductase red1, which could be another D-galactose reductase in addition to xyrA. These genes would explain why the sdhA null mutant was still able to grow on galactitol as well as why the xyrA null mutant is still able to grow on 2 % D-galactose + 0.025 % D-xylose. However, the growth could also be explained by unspecific enzyme activity and thus, further studies would be needed to find out if deh1 or red1 are involved in oxido-reductive D-galactose catabolism.

4.3 Biotechnological applications of L-rhamnose and D-galactose

L-Rhamnose is a relatively rare sugar. Although it is present in plant biomass, the percentage of L-rhamnose is usually well below 1 % (Table 1). There are some exceptions such as green seaweed (Ulva lactuca) which has a L-rhamnose con-
tent around 7% (van der Wal et al. 2013). In the sulphated polysaccharide isolate from the marine green algae *Monostroma latissimum* the L-rhamnose content is even higher comprising 87 mol-%, while the second most abundant monosaccharide, D-xylose, comprises only 6.29 mol-% (Mao et al. 2009). Thus, if algal cell factories become more common in the future, there might be vast amounts of biomass available with exceptionally high L-rhamnose content. In this case, it would be important to also have microbes that are capable to utilise the L-rhamnose among the other biomass sugars and convert them to high value products by biotechnological applications. The product does not need to be one of the natural end products of the pathway (i.e. pyruvate, L-lactaldehyde or L-lactic acid) but it can also be one of the intermediates or a product where the intermediate has been further converted to produce something useful. In the case of a high value product such as a pharmaceutical, the conversion of even a small amount of L-rhamnose could be economically feasible.

Since L-rhamnose is a rare and relatively expensive sugar, the understanding of L-rhamnose catabolism can also be used to prevent its utilisation and to promote its biosynthesis instead. L-Rhamnose is useful and valuable as a precursor for flavouring agents in the food industry. Cocoa pods have L-rhamnose containing polysaccharides (Blakemore et al. 1966, Figueira et al. 1994). Hydrolysed cocoa shell pectin extract has L-rhamnose content between 5 mM and 100 mM and it can be further used as a precursor in the production of concentrate for food and chocolate production (Hansen et al. 2003). Instead of extracting L-rhamnose from cocoa shells or some other plant sources, the alternative option is to produce L-rhamnose by microbes. This has already been done in *Pseudomonas aeruginosa*, which is a good rhamnolipid producer (Ito et al. 1971, Reiling et al. 1986). Rhamnolipids can then be further hydrolysed into L-rhamnose monomers (Linhardt et al. 1989, Giani et al. 1996). It could be possible to find even better L-rhamnose producers, where L-rhamnose could be synthesised as such and one of the essential steps of the production host could be engineered to block the existing L-rhamnose catabolic pathways.

D-galactose is a much more abundant sugar in nature compared to L-rhamnose (Table 1) and hence it would be important to generate microbial cell factories able to also utilise D-galactose if they do not naturally have this ability. There has already been a long term interest for efficiently utilising D-galactose. For example, in *S. cerevisiae*, D-galactose has been used as an efficient carbon source together with D-glucose after an adaptation phase (Ernandes et al. 1992). The GAL gene regulatory network has also been successfully engineered in *S. cerevisiae* resulting in a 41% increase in flux through the D-galactose pathway and thus increased D-galactose consumption (Ostergaard et al. 2000). Different combinations of genetic modifications known to affect D-galactose and D-xylose utilisation were studied to find out how they affect the utilisation of a mixture of carbon sources in *S. cerevisiae* (Garcia Sanchez et al. 2010a). The overexpression of the last step of the Leloir pathway involving the phosphoglucomutase 2, *PGM2*, encoding gene increased the D-galactose uptake (Bro et al. 2005) and enhanced the growth in anaerobic D-galactose fermentation (Garcia Sanchez et al. 2010b). There are also
4. Discussion

several studies where the engineering of the regulatory network has beneficially affected the D-galactose utilisation in *S. cerevisiae* (Ostergaard et al. 2000, Ostergaard et al. 2001, Lee et al. 2011). Although there is no evidence for a functional Leloir pathway in *A. niger*, similar types of approaches could also be applied to increase the natural D-galactose utilisation in the oxido-reductive pathway and possibly make *A. niger* able to grow on sources containing only D-galactose as a carbon source or to increase the transport and D-galactose utilisation rate.

4.4 Glycolic acid production in yeast

Microbial production of glycolic acid had already raised interest before this study as there are several patent applications describing glycolic acid production in *E. coli* through the glyoxylate cycle (Soucaille 2007, Dischert and Soucaille 2010, Dischert et al. 2011a, Dischert et al. 2011b). The engineering involved overexpression of the endogenous *E. coli* glyoxylate reductases *ycdW* and *yiaE* and deletion of the malate synthase genes *aceB* and *glcB*. In addition, other modifications increasing the flux towards glyoxylate were carried out, such as isocitrate lyase, *aceA*, overexpression. This resulted in a production strain which was described to produce 52 g l\(^{-1}\) of glycolic acid from D-glucose in a fed batch bioreactor cultivation (Dischert and Soucaille 2010). An engineered glycolic acid producing *E. coli* strain has also been described recently by Martin et al. (2013) in a study where glycolic acid was an intermediate of the production pathway of 3,4-dihydroxybuturate and 3-hydroxy-γ-butyrolactone. Their engineering approach was very similar to the ones showed in the patent applications although the production pathway and conditions were not as optimised. Thus, 1.4 g l\(^{-1}\) glycolate production was attained from 10 g l\(^{-1}\) D-glucose.

Although glycolic acid is not a common metabolite in proportion to lactic acid, it is still naturally produced by microbes. There are even microbes that are able to naturally produce glycolic acid in high amounts from glycolonitrile or ethylene glycol. However, their industrial use is limited because these pathways are very limited in the substrates they can utilise or the species and production conditions are not well suited for economically feasible bioreactor cultivations. Several microorganisms have been described which are able to produce glycolic acid from ethylene glycol by oxidation to titres over 100 g l\(^{-1}\) when using *Pichia naganishii* or *Rhodotula* sp. (Kataoka et al. 2001). In addition, hydrolysis from glycolonitrile is possible by using *Alcaligenes* sp. soil isolate (He et al. 2010) and some chemolithotrophic iron- and sulphur oxidising bacteria are known to produce glycolic acid by partially unknown metabolic routes in acidic biomining (Nancucheo and Johnson 2010).

4.4.1 Benefits and challenges of a fungal host in organic acid production

In this study, glycolic acid was produced for the first time using a glyoxylate cycle exploiting pathway in a eukaryotic host. Although other production alternatives
have already been described, this particular approach offers several benefits. The route is generally suitable to convert any sugar or C2 compound to glycolic acid since all the required enzymes exist. The yeasts *S. cerevisiae* and *K. lactis*, which were used in this study, are well-known production organisms that have already been used for decades for genetic studies and industrial applications. *K. lactis* is a producer of enzymes for lactose degradation, it is used as a host for heterologous protein production (van Ooyen et al. 2006) and it has also been successfully engineered for D-xylonate production (Nygård et al. 2011). Unlike *S. cerevisiae*, *K. lactis* is a Crabtree-negative yeast so it relies on respiratory metabolism instead of ethanol fermentation. Because of this significant difference in metabolism, *K. lactis* was selected as an alternative host in addition to *S. cerevisiae*. An engineered *S. cerevisiae* strain capable of D-xylose utilisation was selected as the parent strain to allow for an efficient utilisation of lignosellulosic hydrolysates which are rich in C5 sugars. On the other hand, *K. lactis* is naturally capable of utilisation xylose. It was hypothesised that D-xylose would not cause a severe repression of the glyoxylate cycle enzymes like D-glucose does.

The glycolic acid production described earlier in *E. coli* by Soucaille et al. (2007) also involves the use of the glyoxylate cycle but using yeast as a production host has several benefits in organic acid production. Compared to *E. coli*, yeast can tolerate much better low pH conditions, which is an advantage since the contamination risk is reduced and the low pH also reduces the need for neutralisation. In an industrial process, the use of less base results in less undesired salt formation. Lactic acid is a similar example as it has been produced in lactic acid bacteria but for the above mentioned reasons, production at acidic pH has been demonstrated to be advantageous and thus engineered yeast is already used in lactic acid production on an industrial scale (Rajgarhia et al. 2004, Sauer et al. 2010, Miller et al. 2011).

Even though the glyoxylate cycle is present in both eukaryotic microbes and bacteria and the enzymes are the same, the major difference is that in yeasts, the localisation of the enzymes is divided into different compartments of the cell, which requires transport between the cell organelles. For the enzymes directly involved in glycolic acid production, citrate synthase, Cit2p, is peroxisomal and isocitrate lyase, Icl1p, and aconitase, Aco1p, are cytosolic (Kunze et al. 2006). The localisation of the enzymes was not affected in this study but targeting the enzymes into the cytosol could be beneficial as it would decrease the need for transport.

### 4.4.2 Toxicity of glycolic acid

One important and obvious trait for the production host is its tolerance towards the produced product and hence, the toxicity of glycolic acid was tested before proceeding to the strain engineering. Glycolic acid was not very toxic to *S. cerevisiae* or *K. lactis* even at acidic pH; both species are able to grow at glycolic acid concentrations up to 50 g l\(^{-1}\). *K. lactis* is more sensitive at pH 3 but less sensitive at pH 5 compared to *S. cerevisiae* but overall, the tolerance decreased at pH 3 as
4. Discussion

anticipated. The pKa of glycolic acid is 3.83 and at pH lower than that (e.g. pH 3),
it is mainly present in its undissociated form. In the undissociated form, the acid
molecule is uncharged and unlike the dissociated form it freely diffuses across the
membrane causing anion accumulation in the cytoplasm decreasing the intracellular
pH, which further leads to inhibition of glycolysis and results in depletion of ATP
causing restriction of growth (Krebs et al. 1983).

There is not much published data on tolerance towards glycolic acid. A few aci-
dophilic bacteria and one acidophilic archeon were cultivated at pH 2.5-3 and
depending on the species, growth occurred until the glycolic acid concentration
was 1.3 g l\(^{-1}\) for the most sensitive species and 66 g l\(^{-1}\) for the most tolerant spe-
cies (Nancucheo and Johnson 2010). As *Pichia naganishii* and *Rhodotula* sp. are
able to produce high amounts of glycolic acid, it is likely that they are also able to
grow in relatively high glycolic acid concentration even though this was not studied
by Kataoka et al. (2001). However, the pH used in the cultivations was 7.0 and
thus there is no pH benefit over the production method described in *E. coli*.

There are currently no studies on the toxicity caused by glycolic acid in yeast
but the effect of weak organic acids such as acetic acid, benzoic acid, sorbic acid
and lactic acid have been widely studied from the point of view of the cell being
placed in an acidic environment. In the acid production the situation is not exactly
the same as the acid is produced inside the cell and present in the cytosol regard-
less of the membrane diffusion and therefore the responses of the cells might not
be identical. However, the conclusion of the Bioscreen cultivations was that the
toxicity of glycolic acid in both yeast species was not severely preventing cell
growth making the yeasts *S. cerevisiae* and *K. lactis* potentially able to produce
glycolic acid at high titres.

4.4.3 Engineering of the fungal glyoxylate cycle

The glyoxylate cycle offers the possibility to convert all utilisable sugars into glycol-
ic acid as the pyruvate produced during glycolysis can be further metabolised into
acetyl-CoA, which is then fed into the glyoxylate cycle. However, glycolic acid is
not an intermediate of the glyoxylate cycle but an additional enzyme, glyoxylate
reductase, is needed to convert the glyoxylate to glycolic acid. Glyoxylate reduct-
ases are found from prokaryotes to mammals but only a few enzymes and their
corresponding genes have been characterised. Glyoxylate reductases can be
NADPH dependent (EC 1.1.1.79) or NADH dependent (EC 1.1.1.26). Although
usually both co-factors are accepted, the majority of the characterised enzymes
have a strong preference towards NADPH.

In *S. cerevisiae*, two glyoxylate reductase isoforms have been purified
(Tochikura et al. 1979, Fukuda et al. 1980) but only one corresponding gene,*GOR1*, has been identified (Rintala et al. 2007). The biological role of the enzyme
is not clear but as the substrate of the enzymatic conversion, glyoxylate, is known
to be toxic at least for mammalian cells (Poldelski et al. 2001) it might have a role
in preventing accumulation of glyoxylate to the cell as described in plants (Allan et
However, the difference is that in plants, at least the major function of glyoxylate reductase is not to be part of the glyoxylate cycle but the enzyme is involved in the γ-amino butyrate (GABA) pathway acting in plant photorespiration. Glyoxylate and succinic semialdehyde are toxic intermediates of the pathway accumulating in plants during stress (Allan et al. 2009). Two glyoxylate reductases have been identified from *A. thaliana*, a cytosolic GLYR1 (Hoover et al. 2007) and a plastidial GLYR2 (Simpson et al. 2008).

For glycolic acid production, one of the *A. thaliana* glyoxylate reductases, the cytosolic GLYR1, was a promising candidate as it is known to play an important role for the organism and the $K_m$ of 4.5 µM of purified *A. thaliana* GLYR1 (Hoover et al. 2007) was the lowest one among the glyoxylate reductases described in the literature where the corresponding genes have also been characterised. Overall, in addition to the *A. thaliana* enzymes, there are only eight such glyoxylate reductase enzymes: the GOR1 of *S. cerevisiae* (Tochikura et al. 1979, Fukuda et al. 1980, Rintala et al. 2007), the ycdW and yiaE of *E. coli* (Nuñez et al. 2001), the enzymes, which were expressed in the glycolic acid producing *E. coli* strains, gxrA from *Rhizobium etli* (Fauvart et al. 2007), GR from *Thermus thermophilus* HB27 (Ogino et al. 2008), GR from archeon *Thermococcus litoralis* (Ohshima et al. 2001), GR from *Pyrococcus horikoshi* OT3 (Yoshikawa et al. 2007) and GRHPR from human (Cramer et al. 1999). However, these were disregarded due to unfavourable kinetic properties.

Further engineering approaches were carried out in order to increase the flux towards glyoxylate. The malate synthase deletion disrupted the glyoxylate cycle and increased glycolic acid production but as anticipated, it also made the strains unable to grow on ethanol. This was an expected result as the poor growth of *mll* mutant on ethanol media has been shown (Hartig et al. 1992, Kunze et al. 2002). Also, the isocitrate dehydrogenase encoding gene, *IDP2*, was deleted to prevent isocitrate conversion into α-ketoglutarate. The enzyme is cytosolic and suggested to provide α-ketoglutarate for TCA cycle and for cytosolic glutamate synthesis during growth on non-fermentable carbon sources (Haselbeck and McAlister-Henn 1993). However, the importance of the enzyme for the glyoxylate cycle and glycolic acid production is unclear. The deletion of the enzyme was done in combination with overexpression of the isocitrate lyase encoding gene, *ICL1*, in *S. cerevisiae*, which makes it impossible to analyse if the increase in glycolic acid production was exclusively caused by the *ICL1* overexpression or if the *IDP2* deletion also played an important role.

The results suggest that only ethanol is converted to glycolic acid and sugars are used for growth and cell maintenance. Especially in the case of *S. cerevisiae*, a well known ethanol producer, it is presumed that the sugars are converted to ethanol first and then further to glycolic acid, but more studies would be needed to confirm this. As a *D*-xylose utilising *S. cerevisiae* strain was used for the study, the cells can also convert this sugar to ethanol although it is known that *D*-xylose is not as efficiently converted to ethanol as *D*-glucose (Matsushika et al. 2009). From this perspective, *D*-glucose would be a better co-substrate than *D*-xylose. However, by using mixture of *D*-xylose and ethanol the repression of the glyoxylate cycle was
4. Discussion

assumed to be a less severe problem. This was also supported by the isocitrate lyase activity assays where Icl1p was more active when the *S. cerevisiae* cells were grown on D-xylose instead of D-glucose.

*K. lactis* was a significantly better glycolic acid producer compared to *S. cerevisiae*. However, the reasons behind this are not clear as for example the enzymatic activities of the glyoxylate cycle enzymes of these two species were not compared. Based on the results presented in this thesis, it is hard to establish whether the better glycolic acid production capability of *K. lactis* was a consequence of the Crabtree-negative character of the species or if some other trait made *K. lactis* a better glycolic acid producer compared to *S. cerevisiae*.

4.4.4 Regulation of the glyoxylate cycle

Although all the enzymes necessary to convert sugars to glycolic acid do exist in yeasts, the challenge is that the enzymes of the glyoxylate cycle are highly glucose repressed in *S. cerevisiae* and thus the pyruvate is not converted to acetyl-CoA but fermented into ethanol. The first studies on regulation of glyoxylate cycle enzymes in *S. cerevisiae* were carried out by Dunze et al. in 1969 (Dunze et al. 1969) and since then it has been shown that all of the glyoxylate cycle enzymes: isocitrate lyase, Icl1p, (Herrero et al. 1985, Schöler and Schuller 1993),aconitase, Aco1p, (Gangloff et al. 1990), citrate synthase, Cit2p, (Kim et al. 1986), malate synthase, Mls1p, (Hartig et al. 1992) and malate dehydrogenase, Mdh2p, (Minard and McAlister-Henn 1991) are sensitive to carbon catabolite repression. For *K. lactis*, there is not as much published data available but it has been suggested that transcription of the isocitrate lyase is glucose repressed and the gene was expressed on ethanol grown cells (López et al. 2004). However, regulation of carbon metabolism for these two yeast species is different. In *S. cerevisiae*, the isocitrate lyase activity is not only under transcriptional control but post-transcriptional inactivation of the enzyme occurs via phosphorylation (López-Boado et al. 1988). The mechanism is mediated by cAMP-dependent protein kinase catalytic subunits Tpk1p and Tpk2p (Ordiz et al. 1996). In addition, glucose induced proteolytic inactivation is also a mechanism for the Icl1p control (López-Boado et al. 1987). In *K. lactis* instead, no post-transcriptional regulation of the isocitrate lyase was detected (López et al. 2004) and hence it could be assumed that the problem of catabolite repression affecting the glyoxylate cycle enzymes could be more easily solved.

The most important regulator at the transcriptional level of the glyoxylate cycle enzymes is the serine/threonine kinase encoded by *SNF1*, which is conserved among eukaryotes from plants to mammals and required together with the Cat8p transcriptional activator for the utilisation of nonfermentable carbon sources in *S. cerevisiae* and *K. lactis* (Charbon et al. 2004). The difference is that while in *S. cerevisiae* the activity of Snf1p is increased during glucose starvation, the regulation in *K. lactis* is not under carbon catabolite repression at the transcriptional
level and thus Charbon et al. (2004) suggested that post-transcriptional regulation of Cat8p is important in *K. lactis*.

Since the Snf1p is not only controlling the transcription of glucose repressed genes but also has e.g. a broad role in stress tolerance (Sanz 2003) the problem of carbon catabolite repression was attempted to be solved by deletion of Reg1p, which functions as a targeting subunit of the phosphatase complex Reg1Glc7 in *S. cerevisiae* (Sanz et al. 2000). The deletion of *REG1* has been shown to relieve the glucose repression of many genes excluding the gluconeogenic genes (Ludin et al. 1998). However, the deletion of *REG1* did not lead to increased glycolic acid production in the modified *S. cerevisiae* glycolic acid production strain (pGLYR1, *mls1-d1, da1-Δ1, idp2-Δ1::ICL1*). This result suggests that deletion of the *REG1* gene would probably not increase glycolic acid production in any other *S. cerevisiae* strain either, although the isocitrate lyase activity did increase on D-glucose and D-xylose grown cells. Still, the enzyme activity was much higher in the cells grown on the ethanol-glycerol mixture, which might be at least partially explained by glucose induced post-translational inactivation mechanisms. As the glucose repression mechanisms are very diverse, the field offers multiple options for engineering but as the exact mechanisms behind glucose repression are still unclear (Turcotte et al. 2010) and also dependent on the organism, choosing the target for engineering is challenging.
5. Conclusions and future prospects

The fungal oxido-reductive L-rhamnose and D-galactose pathways have been studied. All of the enzymes involved in these pathways have been characterised and the corresponding genes identified. The transporters and transcription factors are still an area requiring more research. Many sugar transporters have been identified but it is not known, which ones are involved in L-rhamnose or D-galactose transport in filamentous fungi. Putative transcription factors were already identified in L-rhamnose work but the identification is currently only based on transcriptome analysis and identification of domains typical to transcription factors. The identification of the genes encoding the enzymes involved in these catabolic routes can be used for the engineering of other organisms. Not all microbes are able to utilise L-rhamnose or D-galactose and thus these enzymes could be heterologously expressed in other species to allow for a broader substrate utilisation capacity.

Glycolic acid production was successfully demonstrated in yeast. A product titre of 15 g l\(^{-1}\) was achieved which is already a promising result, although it is not high enough yet to allow for economically feasible biochemical production. There are still a lot of possibilities to further engineer the yeast strains and thus increase the production. For example, glucose repression is one key issue which should be studied further to find ways to allow the use of sugar substrates. Currently, only ethanol and possibly other C2 substrates can be utilised for glycolic acid production. S. cerevisiae is a well-known ethanol producer and thus it would be possible to first convert sugars into ethanol and only then to glycolic acid but a more direct metabolic conversion route would allow better production yields and probably also faster production rates. As all the glucose repression mechanisms are not fully understood, one option could also be to engineer the whole pathway from sugar to glycolic acid so that all enzymes involved would be expressed under constitutive promoters.

The cofactor availability is an issue that was not studied in this work but will require additional attention in the future. The A. thaliana GLYR1 is a NADPH requiring enzyme and the availability of NADPH might be a limiting factor in glycolic acid production. The deletion of IDP2 can especially lead to a pronounced lack of NADPH as IDP2 is a NADPH producing enzyme (Loftus et al. 1994) which has a significant role in NAPDH production on non-glucose carbon sources such as
oleate and acetate (Minard and McAlister-Henn 2005). Alternative solutions could be to overexpress some other NADPH producing enzyme such as glucose-6-phosphate dehydrogenase (Zwf1p) or a cytosolic aldehyde dehydrogenase (Ald6p) which have been found to be one of the main contributors in cytosolic NADPH production (Grabowska and Chelstowska 2003). The other options would be to find an efficient NADH requiring glyoxylate reductase or to change its cofactor specificity by protein engineering. More studies could also be carried out to determine whether the deletion of isocitrate dehydrogenase is even necessary since the deletion was always done in combination with isocitrate lyase overexpression and thus it is difficult to determine the exact effect of the IDP2 deletion.

Further work is needed to allow for more efficient utilization of both pure substrates and fractions of pre-treated biomass such as hemicellulose hydrolysates. Although an engineered yeast host would be able to catabolise a range of pure sugars, the hydrolysate substrate still offers new challenges. In addition to breaking down the polysaccharide structure of biomass, the thermo-chemical pretreatment step produces inhibitory compounds such as weak acids, furans and phenolic compounds (Parawira and Tekere 2011). Once the production of a chemical such as glycolic acid has been demonstrated with pure substrates the following step is to test and optimise the process with cheaper and more environmentally sustainable substrates. This is not an easy task, especially if the market price for the end product is cheap. For example, ethanol fermentation has been studied for several decades but it is still challenging to economically produce second-generation ethanol made from lignocellulosic biomass (Laluce et al. 2012).

Discovery of new enzyme encoding genes opens more possibilities to engineer and develop microbial cell factories, which can be engineered in order to develop new biochemicals. The work presented in this thesis gives more understanding of L-rhamnose and D-galactose catabolism which can be applied in further strain engineering efforts. These two hexoses are present in hemicellulose and thus it is important to gain understanding of their catabolism.

Biotechnological production of glycolic acid has a good potential for becoming an industrially relevant production process. Although an increase in product titers are necessary and broader substrate utilisation capacity is required of the yeast host, glycolic acid can already be added to the list of promising biochemical candidates where the production has been demonstrated on a lab scale. It is possible, that after a couple of additional years of research, glycolic acid could be among the industrially relevant microbially produced chemicals in addition to e.g. lactic and citric acid. In the future, biotechnology will have a growing importance on the production of everyday life chemicals and thus profound understanding of the different aspects of cell metabolism is needed.
References


Brunner NA, Brinkmann H, Siebers B, Hensel R (1998) NAD+-dependent glycer-
aldehyde-3-phosphate dehydrogenase from Thermoproteus tenax. The first identified archaeal member of the aldehyde dehydrogenase super-
family is a glycolytic enzyme with unusual regulatory properties. J Biol Chem 273: 6149–6156.

Budgen N, Danson MJ (1986) Metabolism of glucose via a modified Entner-


Catabolism of biomass-derived sugars in fungi and metabolic engineering as a tool for organic acid production

Outi Koivistoinen

The use of metabolic engineering as a tool for production of biochemicals and biofuels requires profound understanding of cell metabolism. The pathways for the most abundant and most important hexoses have already been studied quite extensively but it is also important to get a more complete picture of sugar catabolism. In this thesis, catabolic pathways of L-rhamnose and D-galactose were studied in fungi. Both of these hexoses are present in plant biomass, such as in hemicellulose and pectin. Galactoglucomannan, a type of hemicellulose that is especially rich in softwood, is an abundant source of D-galactose. As biotechnology is moving from the usage of edible and easily metabolisable carbon sources towards the increased use of lignocellulosic biomass, it is important to understand how the different sugars can be efficiently turned into valuable biobased products.

Identification of the first fungal L-rhamnose 1-dehydrogenase gene, which codes for the first enzyme of the fungal catabolic L-rhamnose pathway, showed that the protein belongs to a protein family of short-chain alcohol dehydrogenases. Sugar dehydrogenases oxidising a sugar to a sugar acid are not very common in fungi and thus the identification of the L-rhamnose dehydrogenase gene provides more understanding of oxidative sugar catabolism in eukaryotic microbes. Further studies characterising the L-rhamnose cluster in the yeast Scheffersomyces stipitis including the expression of the L-rhamnurate dehydratase in Saccharomyces cerevisiae finalised the biochemical characterisation of the enzymes acting on the pathway. In addition, more understanding of the regulation and evolution of the pathway was gained.

D-Galactose catabolism was studied in the filamentous fungus Aspergillus niger. Two genes coding for the enzymes of the oxido-reductive pathway were identified. Galactitol dehydrogenase is the second enzyme of the pathway converting galactitol to L-xyl-3-hexulose. The galactitol dehydrogenase encoding gene ladB was identified and the deletion of the gene resulted in growth arrest on galactitol indicating that the enzyme is an essential part of the oxido-reductive galactose pathway in fungi. The last step of this pathway converts D-sorbitol to D-fructose by sorbitol dehydrogenase encoded by sdhA gene. Sorbitol dehydrogenase was found to be a medium chain dehydrogenase and transcription analysis suggested that the enzyme is involved in D-galactose and D-sorbitol catabolism.

The thesis also demonstrates how the understanding of cell metabolism can be used to engineer yeast to produce glycolic acid. Glycolic acid is a chemical, which can be used for example in the cosmetic industry and as a precursor for biopolymers. Currently, glycolic acid is produced by chemical synthesis in a process requiring toxic formaldehyde and fossil fuels. Thus, a biochemical production route would be preferable from a sustainability point of view. Yeasts do not produce glycolic acid under normal conditions but it is a desired production host for acid production because of its natural tolerance to low pH conditions. As a proof of concept, pure model substrates, e.g. D-xylose and ethanol, were used as starting materials for glycolic acid production but the knowledge can be further applied to an expanded substrate range such as biomass derived sugars. Already the introduction of a heterologous glyoxylate reductase gene resulted in glycolic acid production in the yeasts S. cerevisiae and Kluyveromyces lactis. Further modifications of the glyoxylate cycle increased the production of glycolic acid and it was successfully produced in bioreactor cultivation.

The challenge of biotechnology is to produce high value products from cheap raw materials in an economically feasible way. This thesis gives more basic understanding to the topic in the form of new information regarding L-rhamnose and D-galactose metabolism in eukaryotic microbes as well as provides an example on how cell metabolism can be engineered in order to turn the cell into a cell factory that is able to produce a useful chemical.
Metabolism of the katabolic pathway of rhamnose and galactose metabolism in Saccharomyces cerevisiae: metabolic engineering, glycolate cycle, glycolic acid, and L-galactose metabolism.

The study investigates the role of the katabolic pathway of rhamnose and galactose in Saccharomyces cerevisiae. The enzyme L-rhamno-1-dehydrogenase is expressed and its activity is investigated. The enzyme is found to be active in the presence of L-rhamnose, but not in the presence of L-galactose, indicating a specific role in the metabolism of rhamnose.

The study also includes the expression of the L-galactose dehydrogenase gene, showing its activity in the presence of L-galactose. The enzyme is found to be active in the presence of both L-rhamnose and L-galactose, indicating a dual role in the metabolism of these sugars.

Overall, the study provides insights into the role of the katabolic pathway of rhamnose and galactose in Saccharomyces cerevisiae, highlighting the specific activities of the enzymes involved.
Catabolism of biomass-derived sugars in fungi and metabolic engineering as a tool for organic acid production

There is a growing interest to find ways to replace petroleum based fuels and chemicals. Alternative production systems are needed, since in the future fossil fuel sources may be scarce and oil prices high. Environmental concerns are also driving research towards greener alternatives, as CO$_2$ and other greenhouse emissions are particularly problematic in the use of non-renewable oil reservoirs. In this thesis, the focus is the study of sugar metabolism in fungi and further engineering of the cells to convert them into microbial cell factories.

Currently, biochemicals are typically produced from corn, sugarcane or other materials with high glucose content. However, it is important to study the metabolism of sugars on a broader scope, so that less easily utilisable sugars could also be efficiently used by microbes. This would allow, for example, the use of non-edible substrates such as lignocellulosic biomass or waste products for the production of fuels and chemicals. In this thesis, two such metabolic pathways have been studied, the L-rhamnose and D-galactose pathways.

This thesis also includes the engineering of a natural metabolic route, the glyoxylate cycle, in order to produce the useful biochemical, glycolic acid. Glycolic acid is used for example in cosmetics and can also be used as a precursor for poly-glycolic acid (PGA), which has excellent gas barrier properties. Two yeast species, Saccharomyces cerevisiae and Kluyveromyces lactis, were engineered and glycolic acid production was successfully demonstrated in bioreactor cultivation.